The autocrine and paracrine regulation of endothelial cell function by F-Prostanoid receptor signalling.

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Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Margaret Keightley

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Publications and presentations

Data from chapter 4 and 5 has been included in the following article:
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Abstract

Endometrial adenocarcinoma, originating from the glandular epithelial cells of the uterine endometrial lining, is one of the most prevalent cancers amongst women in the Western world. The prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor (FP) is upregulated in endometrial adenocarcinoma. A previous microarray analysis of endometrial adenocarcinoma cells (Ishikawa) identified numerous targets of $PGF_{2\alpha}$-FP signalling including angiogenic factors, VEGF-A, FGF-2, CXCL1 and CXCL8 and antiangiogenic factors ADAMTS1. The regulation of VEGF-A, FGF-2, CXCL1 and CXCL8 was confirmed by previous studies using an in vitro model system, of Ishikawa cells stably expressing the FP receptor to levels observed in cancer (FPS cells). In this thesis, ADAMTS1 expression was found to be upregulated in endometrial adenocarcinoma samples compared to normal endometrium. Using FPS cells, ADAMTS1 expression was regulated in an extracellular signal regulated kinase 1/2 (ERK1/2) independent manner involving activation of nuclear factor of activated T cells (NFAT). Angiogenic and antiangiogenic proteins secreted by epithelial cells, in response to $PGF_{2\alpha}$-FP receptor signalling, could therefore regulate vascular function in a paracrine manner. Hence this thesis examines the role of angiogenic factors FGF2, CXCL1 and CXCL8, secreted into $PGF_{2\alpha}$-treated FPS cell conditioned medium (P CM), in the regulation of endothelial cell function in vitro.

Firstly, using an in vitro model system, treatment of human umbilical vein endothelial cells (HUVECs) with P CM increased endothelial network formation and proliferation, compared to control CM. Immunoneutralisation of FGF2, CXCL1 and CXCL8 from the P CM reduced endothelial cell network formation and proliferation ($P<0.05$). In addition, inhibition of their receptors (FGFR1 and CXCR2) with chemical antagonists decreased endothelial cell network formation and proliferation ($P<0.05$) in response to treatment with P CM. This indicates that FGF2, CXCL1 and CXCL8 are paracrine effectors of FP-mediated endothelial cell network formation and proliferation.

Next, the mechanisms by which FGF2 regulates P CM-induced endothelial cell network formation and proliferation were investigated. Using specific inhibitors of cell signalling, FGF2-FGFR1 was found to regulate endothelial cell proliferation via the mTOR pathway. In contrast, FGF2-FGFR1 signalling mediated endothelial cell network formation via the regulation of COX-2 expression and $PGF_{2\alpha}$ synthesis in endothelial cells.

Angiogenesis is maintained by a balance of pro-and antiangiogenic factors. Hence, concomitantly with the upregulation of proangiogenic factors, antiangiogenic proteins ADAMTS1 and regulator of calcineurin 1 (RCAN1) were upregulated by P CM treatment of HUVECs. They were subsequently shown to limit endothelial cell network formation and proliferation in response to P CM.

Finally, the role of $PGF_{2\alpha}$ in angiogenesis was investigated using two in vivo models. $PGF_{2\alpha}$ treatment did not increase angiogenesis in a sponge matrigel mouse model. In a xenograft mouse model, $PGF_{2\alpha}$-FP signalling increased expression of angiogenic factors in human epithelial cells and mouse stroma but this did not enhance microvessel density.

Taken together, this thesis had highlighted that $PGF_{2\alpha}$-FP receptor signalling stimulates expression of pro-and antiangiogenic factors that in turn regulate endothelial cell function. However, in vivo studies demonstrate that $PGF_{2\alpha}$-FP receptor interaction does not impact on the level of angiogenesis but may control other aspects of vascular function.

Abbreviations	
Ad	Adenovirus
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin repeat 1
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
CaM	Calmodulin
CAM	Chick chorioallantoic membrane
cAMP	Adenosine 3',5'- cuclic monophosphate
CM	Conditioned medium
Cn	Calcineurin
COX	Cyclooxygenase
CsA	Cyclosporin A
CXCL1	CXC chemokine 1 (Growth regulated oncogene α; GROα)
CXCL8	CXC chemokine 8 (Interleukin 8; IL8)
CXCR2	CXC chemokine receptor 2
cPLA2α	Cytosolic phospholipase A2
c-Src	Cellular-Src
DAB	3,3'- diaminobenzidine
DMEM	Dulbeccos modified eagle medium
DNA	Deoxyribonucleic acid
EBM1%	Endothelial basal medium containing 1% FBS
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM	Endothelial growth medium
ELISA	Enzyme linked immunosorbent assay
EP	E-Prostanoid receptor

EPC	Endothelial progenitor cell
ERK1/2	Extracellular signal regulated kinase 1/2
FBS	Foetal bovine serum
FGF2	Fibroblast growth factor 2
FGFR1	Fibroblast growth factor receptor 1
FP	F- Prostanoid receptor
FP P CM	$PGF_{2\alpha}$- treated FPS cell conditioned medium
FP V CM	Vehicle- treated FPS cell conditioned medium
GPCR	G-protein coupled receptor
GTP	Guanidine triphosphate
HB-EGF	Heparin binding EGF-like growth factor
HBSS	Hank's buffer saline solution
HDAC	Histone deacetylase
HEECs	Human endometrial endothelial cells
HMECs	Human microvascular endothelial cells
HSECs	Human endometrial stromal cells
HRT	Hormone replacement therapy
HUVEC	Human umbilical vein endothelial cell
HSPG	Heparin sulphate proteoglycan
IgG	Immunoglobulin G
IGM	Intussusceptive microvascular growth
IP	Prostacyclin receptor
IP3	Inositol-1,4,5- triphosphate
kDa	Kilodaltons
LMW	Low molecular weight
lv	Lentivirus
MAPK	Mitogen-activated protein kinase
MEK1/2	Mitogen-activated protein kinase kinase/ extracellular regulated kinase kinase 1/2
MMP	Matrix metalloproteinase
MOX	Methyloxyamine buffer
mTOR	Mammalian target of rapamycin

MVD	Microvessel density
nM	Nano molar
NFAT	Nuclear factor of activated T-cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGES	Prostaglandin E synthase
PGFS	Prostaglandin F synthase
PI3K	Phosphoinositidel-3-kinase
PIP	Phosphatidylinositol
PKC	Protein kinase C
PLC	Phospholipase C
PPAR	Peroxisome proliferator activated receptor
PROK	Prokineticin
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
QPCR	Quantitative-RT-PCR
RCAN	Regulator of calcineurin
RGD	Arg – Gly - Asp
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Real time- polymerase chain reaction
scr	scrambled
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFK	Src-family protein tyrosine kinase
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinases

TNF	Tumour necrosis factor
TNS	Trypsin neutralising solution
TRI	Total RNA isolation
µM	Micro molar
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VM	Vascular mimicry
VWF	Von Willebrand Factor
WT	Wild type
WT P CM	$PGF_{2\alpha}$ treated WT Ishikawa cell conditioned medium
WT V CM	Vehicle treated WT Ishikawa cell conditioned medium

List of Figures

List of Tables

1 Literature Review.

1.1 Introduction

This chapter intends to review the literature in the context of endometrial cancer, prostaglandins and angiogenesis. It will summarise the multitude of data relating to the role of proangiogenic and antiangiogenic factors and the signal transduction pathways regulating endothelial cell function. Endometrial cancer is the most common gynaecological cancer among women in the Western world. Therefore, effective antiangiogenic therapy in endometrial cancer could provide curative and palliative care for thousands of women. In order to identify effective targets of antiangiogenic therapy in women suffering from endometrial cancer, the pathways regulating its initiation and progression must be elucidated.

1.2 Endometrial adenocarcinoma

Endometrial adenocarcinoma is the fourth most common cancer and the most common gynaecological cancer amongst women in the Western world. In 2009 the American Cancer Society estimated that there would be 42,106 new cases of endometrial cancer representing 6% of total new cancer cases for the year (Jemal et al., 2009). In addition, it was estimated that 7,780 deaths will occur from endometrial cancer cases contributing to 3% of total female deaths from cancer in a year (Jemal et al., 2009). The five year survival rate for endometrial cancer is approximately 83% for all races (Jemal et al., 2008a). However, the most effective form of treatment in use is the surgical removal of the uterus, by hysterectomy or laparoscopy. Perhaps due to the lack of new treatments for endometrial cancer, the five year survival rates have not improved over the last 30 years (Jemal et al., 2008a).

More than 80% of endometrial cancers are endometrial adenocarcinomas which originate from the endometrial lining. There are a variety of methods to group endometrial cancers for example; endometrial adenocarcinomas can be defined as two different types, Type 1 which are well, moderately or poorly differentiated

endometrioid cancers under oestrogen control (Doll et al., 2008). Type 2 endometrial cancers are poorly differentiated, non-endometrioid, papillary serous carcinomas (Doll et al., 2008). These two types of endometrial adenocarcinoma appear to have distinct gene expression patterns for example, COX-1 was found to be upregulated in papillary serous carcinomas compared to endometrioid serous carcinomas (Maxwell et al., 2005). In 1997 it was found that loss of function mutations in phosphatase and tensin homolog (PTEN), an essential lipid phosphatase, were associated with 55% of endometrial cancers, more so than other cancers such as gastric, colorectal and pancreatic cancer (Kong et al., 1997). Further studies have found that Type 1 endometrial carcinomas often exhibit a PTEN mutation whereas Type 2 endometrial carcinomas do not commonly exhibit a PTEN mutation and more often have mutated TP53 gene which encodes the master cell cycle regulator p53 (Doll et al., 2008) (Brosh and Rotter, 2009; Okamoto et al., 1991). Approximately 2-4% of endometrial cancers are sarcomas which originate from the smooth muscle of uterine wall called mixed mullerian tumours (Maxwell et al., 2005). These mixed mullerian tumours are aggressive and associated with a poor prognosis (Maxwell et al., 2005). This thesis will concentrate on endometrial adenocarcinomas of Type 1 as the model cell line used in this thesis is the well differentiated Ishikawa cell line. In addition, Type 1 endometrioid, endometrial adenocarcinoma biopsy samples will be subdivided into well (grade 1), moderately (grade 2) and poorly (grade 3) differentiated samples for analysis in this study. However, in spite of the differences in gene expression between endometrial cancer types, they share clinical similarities in their association with obesity, race, oestrogen and tamoxifen (Doll et al., 2008). Despite past and present research, the processes controlling the initiation and progression of endometrial adenocarcinoma are poorly understood. Further investigations into the mechanisms controlling endometrial adenocarcinoma tumourigenesis are required in order to develop new therapeutic strategies.

1.2.1 Endometrial adenocarcinoma in relation to the normal endometrium

1.2.2 Normal endometrium-form and function

The normal human endometrium is a dynamic tissue of the uterine corpus undergoing continuous menstrual cycles of proliferation, differentiation, breakdown and repair in the absence of pregnancy (Gargett and Rogers, 2001). The average menstrual cycle length is between 28 and 32 days although this varies with age (Chiazze et al., 1968). The menstrual cycle can be separated into proliferative and secretory stages with each divided into early, mid and late stages. The progression of the menstrual cycle stages is under hormonal control mediated through the expression of molecular effectors such as growth factors (Jabbour et al., 2006a). The cycle begins after menstruation, menses, the breakdown and shedding of the functional layer of the endometrium. Rapid proliferation of the functionalis is required to replace the tissue lost. This is stimulated by the circulating ovarian steroid oestrogen. The proliferative effects of oestrogen have been examined in a variety of cell types as the regeneration of the functionalis requires the proliferation of epithelial and stromal cells, including the endothelial cells lining blood vessels (Albrecht et al., 2003a; Albrecht et al., 2003b; Fujimoto et al., 1997; Jabbour et al., 2006a).

Following the late proliferative stage is the early secretory stage during which the endometrium is prepared for embryo implantation (Jabbour et al., 2006a). The rise in progesterone levels counteracts the oestrogenic proliferative effects enabling cellular differentiation, although there are still areas of growth around the spiral arteries (Jabbour et al., 2006a). The main blood supply is provided by the spiral arterioles which coil in the newly formed functionalis layer to present a larger surface area for the exchange of oxygen and nutrients. The subepithelial capillary plexus in the basalis, located just below the functionalis, also develops as an essential blood supply that reaches maturity in the early-mid secretory phase (Gargett and Rogers, 2001; Krikun et al., 2004). The endometrium is most receptive to embryo

attachment in the early-mid secretory phase (Jabbour et al., 2006a). In the event of the attachment of an embryo, the endometrium undergoes decidualisation, a special differentiation event controlled by crosstalk between the embryo and endometrium (Jabbour et al., 2006a). Without embryonic implantation a drop in progesterone levels causes an increase in the expression of inflammatory mediators in the endometrium, resulting in breakdown and shedding of the upper third layer of the endometrium thereby completing the menstrual cycle (Jabbour et al., 2006a).

1.2.3 Factors influencing the transition from normal to diseased state

Endometrial adenocarcinoma occurs most commonly in post-menopausal women (Amant et al., 2005). Interestingly, a later onset of menopause, after the age of 55, is associated with a 79% increased risk of endometrial cancer, compared to women whose menopause occurred by age 45 (Setiawan et al., 2007). The occurrence of endometrial adenocarcinoma in post-menopausal women has been shown to be facilitated by long term use of oestrogen only hormone replacement treatment (HRT) (Pike et al., 1997). This association led to the use of the combined HRT, oestrogen with progesterone, which decreases the risk of HRT inducing endometrial adenocarcinoma (Pike et al., 1997). The unopposed oestrogen hypothesis was proposed in the 1980s to increase the mitotic index of the endometrium thereby increasing the possibility of cell mutations and tumour growth (Zeleniuch-Jacquotte et al., 2001).

Unopposed oestrogen may also be the cause of the increased incidence of endometrial cancer in overweight individuals (Calle and Kaaks, 2004; Zeleniuch-Jacquotte et al., 2001). In 1949 a cohort study investigating endometrial cancer found that there was an increased incidence of endometrial cancer in overweight patients (Palmer et al., 1949). Since this latter study, many more studies have confirmed the association of endometrial cancer with obesity. Some studies have suggested that obesity may account for approximately 40% of the incidences of endometrial cancer (Bergström et al., 2001; Calle and Kaaks, 2004). Diabetes, an

obesity associated disease has also been linked to endometrial cancer further suggesting that a disruption in hormonal control may be critical to the onset of endometrial cancer (Fig. 1)(Calle and Kaaks, 2004). In type 2 diabetes and obesity, increased levels of insulin are produced to counteract the development of insulin resistance. The increased levels of insulin cause a decrease in the production of insulin-like growth factor binding protein 1 (IGFBP1). This leads to an increase in circulating levels of Insulin like growth factor (IGF). IGF is a growth factor that can cause the proliferation of many cell types including endothelial cells and cancer cells (Pollak, 2008). In endometrial cancer increased levels of IGF1 are observed (Soufla et al., 2008). Obesity in post menopausal women can also cause an increase in aromatase levels causing higher levels of active oestrogen to be produced (Zeleniuch-Jacquotte et al., 2001). Oestrogen, when unopposed by progesterone can promote endometrial cancer, partly through the increased production of IGF1 (Calle and Kaaks, 2004; Zeleniuch-Jacquotte et al., 2001). In pre-menopausal women a loss of progesterone production, due to ovarian androgen production or anovulation, can result in a decrease in IGFBP1 synthesis which may increase the risk of endometrial cancer (Calle and Kaaks, 2004). It is therefore clear that obesity, diabetes and endometrial cancer are inherently linked through their relationship with oestrogen, progesterone and IGF (Fig. 1).

IGF is not the only effector of oestrogen-regulated endometrial cancer. In addition, PAX2 is a transcription factor whose expression is under the control of oestrogen and tamoxifen (Shang, 2006). The induction of PAX2 expression by oestrogen and tamoxifen has been found to enhance in vitro endometrial cancer cell proliferation and in vivo endometrial cancer xenograft growth (Wu et al., 2005). During development PAX family proteins are widely expressed however, in most adult tissue their expression is switched off by methylation and histone deacetylase (HDAC) binding (Shang, 2006). In endometrial cancer however, the expression of PAX2 is high due to loss of methylation along with the HDAC1 binding complex in the Pax gene promoter (Wu et al., 2005). Incidentally, in a different study, immunohistochemical analysis of endometrial cancer suggested that the expression

of HDAC1 was decreased compared to normal endometrium, suggesting that HDAC1 along with Pax are involved in the epigenetic control of endometrial cancer (Krusche et al., 2007). These data indicate that PAX2 is a molecular effector of oestrogen and tamoxifen in the progression of endometrial cancer (Shang, 2006).

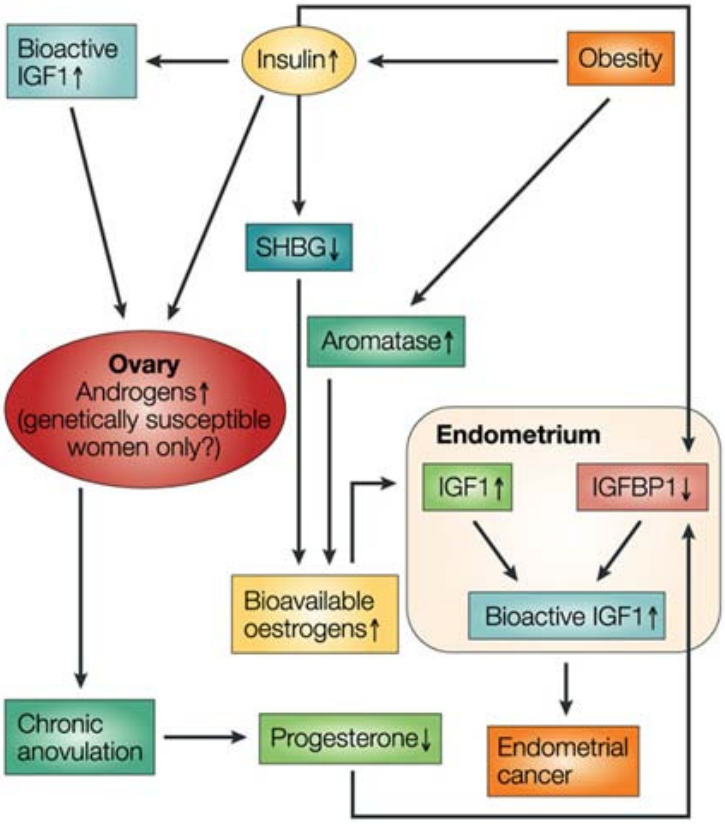


Fig. 1 The relationship between diabetes, obesity and endometrial cancer. Adapted from Calle et al. (2004). High insulin levels seen in patients with type 2 diabetes and obesity can increase in IGF levels through the promotion of bioavaliable oestrogen. In the endometrium an increase in IGF can promote tumour cell proliferation. Pre-menopausal women may be genetically predisposed these pathways. Insulin can also increase sex hormone binding globulin (SHBG) which increases the bioavailability of oestrogens.

In addition to molecular effectors, other regulators may be involved in oestrogen-regulated endometrial cancer for example, tamoxifen-bound oestrogen receptor can interact with p160 steroid receptor coactivators (SRC) (Shang and Brown, 2002; Xu et al., 2009a). There are three forms of SRC 1, 2 and 3 of which SRC3 expression has been found to be increased in endometrial cancer (Balmer et al., 2006; Xu et al.,

2009a). The interaction of oestrogen and progesterone receptors with SRC enables SRC to modulate downstream signalling pathways from the receptors (Balmer et al., 2006). For example, SRC3 is involved in EGFR activation and thereby stimulates the ERK1/2 pathway involved in proliferation (Xu et al., 2009a). Interestingly, Shang et al. found that in Ishikawa cells treated with tamoxifen, IGF-1 upregulation was inhibited by SRC-1 siRNA (Shang and Brown, 2002).

The incidence of endometrial cancer is less in African American, Native Hawaiians, Japanese Americans, and Latina women compared to Caucasian American women however the reasons underlying this difference are not clear (Setiawan et al., 2007). In the US there is a decreased survival rate for endometrial cancer in the African female population compared to the Caucasian female population (Jemal et al., 2008b). Evidence suggests that this difference is not due to any difference in treatments but is due to a higher occurrence of poorly differentiated endometrial adenocarcinoma in the African female population suggesting that the more serious disease state is responsible for the decrease in survival rate (Maxwell et al., 2006).

Other factors that have been associated with endometrial cancer risk are nulliparity and having a first-degree relative with endometrial cancer (Amant et al., 2005). In contrast, oral contraceptive, combined oestrogen and progesterone, use may decrease the risk of endometrial cancer (Amant et al., 2005; Calle and Kaaks, 2004). Also, physical activity is known to decrease the risk of many cancer types including breast cancer and endometrial cancer (Calle and Kaaks, 2004; McTiernan, 2008). Due to the strong link between endometrial cancer and obesity it is evident that physical activity associated with weight loss and a reduction in bioavailable oestrogens may help to decrease an individuals risk of endometrial cancer (Calle and Kaaks, 2004; McTiernan, 2008). In a slight contradiction to the healthy lifestyle, smoking may also decrease the bioavailability of oestrogens and is associated with a decreased risk of endometrial cancer (Amant et al., 2005). Interestingly, a recent population based cohort study suggested that sun exposure, including sun-bed use more than three times a year, may decrease the risk of endometrial cancer by as much as 40%

(Epstein et al., 2009). Epstein et al. hypothesise that the decreased risk of endometrial cancer could be a result of increasing vitamin D levels although as yet there is little evidence for the link between vitamin D deficiency and endometrial cancer (Epstein et al., 2009; McCullough et al., 2008).

1.2.4 Current treatments for endometrial adenocarcinoma.

Patients with localised endometrial adenocarcinoma are treated with standard treatment of hysterectomy with or without radiotherapy (Creutzberg et al., 2000). For patients with regional and distant metastases the standard treatment is inadequate. Progestrone therapy has been investigated in cancers however it was only found to have a beneficial effect if patient cancers expressed the receptors for oestrogen and progesterone (Amant et al., 2005). Alternatively, a phase 3 trial of a three-drug treatment of doxorubicin, cisplatin and paclitaxel (TAP) showed improved survival rates compared to a two-drug treatment of doxorubicin and cisplatin (AP) (Fleming et al., 2004). Unfortunately, the TAP treatment was associated with an increase in peripheral sensory neuropathy due to neurotoxicity (Fleming et al., 2004). Combinations of other drugs including paclitaxel and platinum may show as great affects as TAP without the neurotoxicity (Amant et al., 2005). A more recent trial found that AP treatment of higher grade cancers significantly increased survival compared to whole abdomen radiation (Randall et al., 2006). Due to this, chemotherapy is now used along side radiation in some advanced cases of endometrial cancer (Randall et al., 2006). No molecular therapies are approved for the treatment of endometrial adenocarcinoma however, the tyrosine kinase inhibitor sunitinib, in combination with radiotherapy, is currently in phase 1 clinical trials for cancers including endometrial adenocarcinomas. Sunitinib inhibits the angiogenic signalling of tyrosine kinase receptors VEGFR, PDGFRb and c-kit (Ellis and Hicklin, 2008). In vivo experiments found that sunitinib treatment reduced tumour xenograft growth and normalised tumour vasculature (Ellis and Hicklin, 2008) (Hillman et al., 2009). Therefore, sunitinib could enhance the delivery of chemotherapies (Hillman et al., 2009). Inhibition of sunitinib in colorectal cancer

trials increased survival rates hence, sunitinib could be effectively used to treat other cancers (Ellis and Hicklin, 2008).

It has recently been suggested that targeting of the biolipid hormone receptors, prostaglandin receptors, could be used to treat cancers including endometrial cancer (Jain et al., 2008; Sales et al., 2007). However, the signalling pathways and cell functions controlled by prostaglandin receptor signalling must first be thoroughly investigated.

1.3 Prostaglandins

Prostaglandins are bioactive lipids synthesised from arachidonic acid (AA) by steps involving cyclooxygenase (COX) enzymes (Dubois et al., 1998). COX-1 and COX-2 both regulate prostaglandin synthesis (Dubois et al., 1998). COX-1 is a constitutively active enzyme responsible for basal levels of prostaglandins and COX-2 is considered an inducible enzyme stimulated by growth factors and tumour promoters (Cha et al., 2005). There are four main prostaglandins, PGD_2, $PGF_{2\alpha}$, PGI_2 and PGE_2 and seven corresponding receptors, DP, FP, IP and EP1-4 (Jabbour and Sales, 2004). Prostaglandin receptors belong to the group of G-protein coupled receptors (GPCRs). Once synthesised, prostaglandins can be transported out of the cell, via a prostaglandin transporter (PGT) situated in the cell membrane, and act in an autocrine/paracrine manner (Jabbour and Sales, 2004).

1.3.1 Biological action of prostaglandins PLA_2 and AA.

Phospholipase A_2 (PLA_2) family of enzymes are involved in the rate-limiting step of arachidonic acid (AA) release (Nakanishi and Rosenberg, 2006). For example, cytoplasmic PLA_2 ($cPLA_2$) translocates to the intracellular membrane upon agonist stimulation when intracellular calcium levels are elevated (Alfranca et al., 2006). Once at the intracellular membrane, $cPLA_2$ cleaves phospholipids containing AA (Fig.2) (Nakanishi and Rosenberg, 2006).

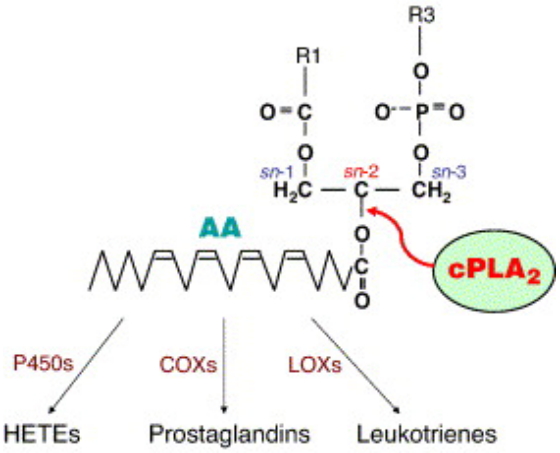


Fig.2. Biological action of cytoplasmic phospholipase A_2 ($cPLA_2$) on arachidonic acid(AA) release. Adapted from Nakanishi et al. (2006).

In the proximity of cyclooxygenases (COX), AA is metabolised into prostaglandin precursors: prostaglandin endoperoxide G_2 (PGG_2) and prostaglandin endoperoxide H_2 (PGH_2) (Nakanishi and Rosenberg, 2006). Recent research by Herbert et al. found that the spatiotemporal co-localisation of $cPLA_2$ with COX-2 at the intracellular membrane is crucial for mediating prostaglandin production in endothelial cells (Herbert et al., 2007). Consequently, sequestration of $cPLA_2$ at the Golgi apparatus can prevent $cPLA_2$ cleavage of AA and hence inhibit prostaglandin production (Herbert et al., 2007). $cPLA_2$ knockout mice have suboptimal production of prostaglandins, including prostaglandin $F_{2\alpha}$, and reduced fertility due to impaired implantation and embryogenesis (Bonventre et al., 1997; Nakanishi and Rosenberg, 2006). The negative effects of $cPLA_2$ knockout on implantation in the mouse uterus can be partially rescued by the addition of prostaglandin E_2 (Song et al., 2002). Interestingly, in $cPLA_2$ striated muscle knockout cells there is exaggerated IGF-1 signalling suggesting that $cPLA_2$ is required for the negative regulation of IGF-1 (Haq et al., 2003). In the suggested mechanism for this, arachidonic acid recruits protein kinase Cζ (PKCζ) to the intracellular membrane and increases the interaction of PKCζ with phosphate-dependent kinase-1 (PDK-1), thereby enhancing PKCζ activation by PDK-1 phosphorylation (Haq et al., 2003). PKCζ is a negative regulator of IGF-1 signalling and can inhibit ERK1/2 phosphorylation stimulated by IGF-1 (Haq et al., 2003). Therefore, the regulation of AA by PLA_2 is critical for

determining downstream signalling regulating many physiological processes (Nakanishi and Rosenberg, 2006). PLA_2 expression is increased in some types of cancer including prostate cancer where it is being considered as a possible therapeutic target for cancer treatment (Patel et al., 2008).

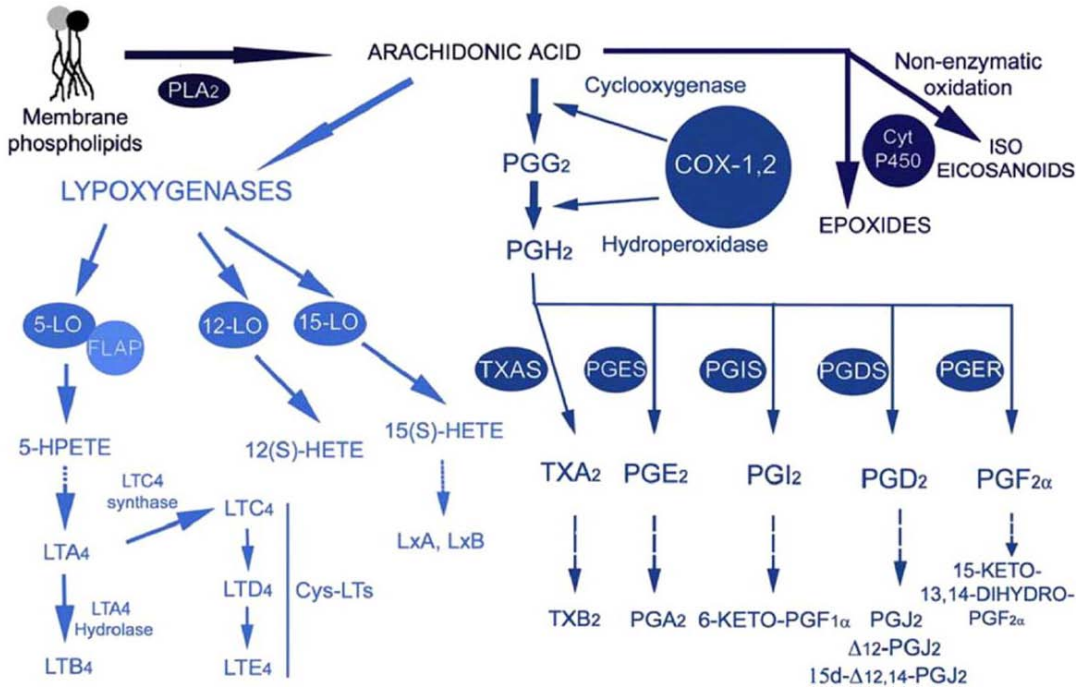


Fig. 3 Schematic of the arachidonic acid (AA) metabolic pathway. Adapted from Alfranca et al.(2006).

In addition to prostaglandin production, AA can be converted by lipoxygenases (LOX) to HETE and leukotrienes (Lötzer et al., 2007). Additionally, in a non-enzymatic process involving cytochrome P450, AA can also oxidise into iso-eicosanoids and epoxides (Alfranca et al., 2006). A by-product of AA metabolism is lysophosphatidic acid (LPA). LPA and its metabolite 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) are known to be involved in the regulation of inflammation and cancer (Nakanishi and Rosenberg, 2006). This thesis however, will focus on the cyclooxygenases and their prostaglandin products.

1.3.2 Cyclooxygenases 1 and 2

There are three isoforms of cyclooxygenase (COX)-1, -2 and the splice variant of COX-1, COX-3, but of these COX-1 and COX-2 have been most extensively

characterised (Dubois et al., 1998; Kam and So, 2009). COX enzymes catalyse the production of AA to prostaglandin endoperoxide G_2 (PGG_2) and from PGG_2 to prostaglandin endoperoxide H_2 (PGH_2). Interest in COX began in the early 1970's when researchers discovered that aspirin inhibition of COX prevented PG secretion. Originally identified in during the study of anaphylaxis in guinea pig lungs, RSC (rabbit aorta contracting substance now called COX) was found to be inhibited by aspirin (acetylsalicylic acid) and indomethacin (Piper and Vane, 1969). Following further studies Vane et al. suggested that COX was responsible for the production of prostaglandins and the target of aspirin inhibition (Vane, 1971). Aspirin, along with other COX inhibitors, is a non-steroidal anti-inflammatory drug (NSAID) that inhibits both COX-1 and COX-2.

COX-1 is expressed in most tissue under basal conditions and is generally thought to be responsible for maintenance of basal levels of prostaglandins regulating homeostasis (Cha and DuBois, 2007). In contrast, COX-2 expression is generally absent in basal tissue conditions but increases in response to external stimuli (Cha and DuBois, 2007). As an inducible enzyme, COX-2 is often elevated during inflammation and cancer (Dubois et al., 1998). Due to this, recent research has investigated the effects of specific COX-2 inhibitors in the resolution of diseases (Cha and DuBois, 2007).

The use of cyclooxygenase inhibitors in therapeutic treatment met with a hurdle when in 2004 the COX-2 inhibitor rofecoxib (Vioxx), used in a clinical trial to prevent colorectal adenoma, was found to cause cardiovascular problems including increased blood pressure (William et al., 2009). This is due to the fact that COX enzymes are essential for normal vascular function and homeostasis through regulating the production of a range of different prostaglandins (Dubois et al., 1998). Vioxx was immediately withdrawn from the market by its producer Merck, a step that affected thousands of people in the UK and US. Henceforth, new research into the targeting of other members of the COX-PG cascade, such as prostaglandin

synthases and prostaglandin receptors, may identify alternative therapeutic targets (Cha and DuBois, 2007).

1.3.3 Prostaglandin synthases

Isomerisation of prostaglandin endoperoxide H_2 (PGH_2) to prostaglandin E_2, $F_{2\alpha}$, prostacyclin (PGI_2) or D_2 is catalysed by prostaglandin synthase (PGS) enzymes (Murakami et al., 2002). In addition, thromboxane endoperoxide synthase catalyses the production of thromboxane (TXA_2) from PGH_2 (Murakami et al., 2002). The prostaglandin produced is determined by the specific PGS that processes PGH_2 for example, prostaglandin E_2 is produced by prostaglandin E_2 synthase (PGES) activity and prostaglandin $F_{2\alpha}$ is produced by prostaglandin $F_{2\alpha}$ synthases (PGFS). In fact there are three isoforms of PGES but of these cytosolic PGES (cPGES) and membrane-bound PGES (mPGES) have been most extensively studied (Murakami et al., 2002). cPGES is ubiquitously expressed and catalyses the products of COX-1 whereas mPGES is inducible and functionally coupled to COX-2 (Murakami et al., 2002; Murakami et al., 2000). $PGF_{2\alpha}$ formation by PGFS can occur in three ways, from PGH_2 catalysed by 9,11-endoperoxide reductase activity, from PGD_2 catalysed by 11-ketoreductase or from PGE_2 catalysed by 9-ketoreductase (Watanabe, 2002). Human placental $PGF_{2\alpha}$ synthase (AKR1B1) and human lung $PGF_{2\alpha}$ synthase (AKR1C3) are two aldoketoreductase enzymes that synthesise $PGF_{2\alpha}$ (Watanabe, 2002). There are three forms of PGFS that have been identified in bovine endometrium, liver and lung cells (Waclawik et al., 2006). PGFS has been found to be expressed in the pig endometrium where it is localised to the glandular epithelial cells (Waclawik et al., 2006). In bovine endometrial stromal cells, the presence of both oestrogen and progesterone was found to upregulate PGFS expression (Xiao et al., 1998). In 1978 Marcus et al. found that prostaglandins E_2, $F_{2\alpha}$ and D_2 also spontaneously formed from PGH_2 via non-enzymatic oxidation whereas prostacyclin formation was enzymatic (Marcus et al., 1978).

1.3.4 Prostaglandins $F_{2\alpha}$ and E_2

Prostaglandin F_2 ($PGF_{2\alpha}$) is a potent regulator of reproductive function involved in promoting luteolysis, parturition, myometrial contractions and cervical ripening (Blatchley and Donovan, 1969). More is known about the role of $PGF_{2\alpha}$ in mammalian species such as sheep, goats and cows than humans (Johnson, 2001). Originally prostaglandin $F_{2\alpha}$ secretion was found to be associated with luteolysis in guinea pigs (Blatchley and Donovan, 1969). Prostaglandin E_2 (PGE_2) has a similar role to $PGF_{2\alpha}$ in the female reproductive system although it is luteotrophic. However, in comparison to $PGF_{2\alpha}$, much more is known about the role of PGE_2 in cell migration, proliferation, apoptosis, vascular formation and cancer.

As prostaglandins regulate reproductive function in many species they therefore have multi-species importance. For example, in the bovine endometrium, prostaglandin E_2 secretion is increased in endometrial infection, endometritis, upon activation of toll-like receptor 4 (TLR4) signalling by bacterial LPS (Herath et al., 2006). The increase in PGE_2 may be due to PLA_2 elevation, rather than an increase in PGES, and can disrupt the ovarian cycle and luteolysis (Herath et al., 2006; Herath et al., 2009). In the farming industry, the effects of endomitritis on cattle reproduction can be devastating due to its detrimental effects on pregnancy and birth rates (Herath et al., 2006). Therefore, research into prostaglandin signalling pathways could have beneficial effects for many species.

One important species dependent difference of the $PGF_{2\alpha}$ receptor, FP receptor, is that the bovine and ovine FP receptor has at least two different splice variants including FP_A and FP_B. Fujino et al. found that the downstream signalling of these two isoforms varies for example, $PGF_{2\alpha}$ can stimulate TNF-alpha promoter activity and T-cell factor (Tcf)/β-catenin signalling through the FP_B but not the FP_A receptor in HEK-293 cells stably expressing either the ovine FP_A or FP_B receptors (Fujino and Regan, 2004; Fujino et al., 2004). In the humans, as well as the hFP_A isoform, the splice variant hFP_S was identified by Regan et al. and is expressed in vascular endothelial, trophoblast, and decidual cells from human placenta, although its

functional relevance is unknown (Vielhauer et al., 2004). This thesis will concentrate on human $PGF_{2\alpha}$-FP signalling and therefore focus on the full length human FP receptor.

Both prostaglandin E_2 and $F_{2\alpha}$ are implicated in tumourigenesis. In colorectal cancer, prostaglandins E_2 has been found to be elevated (Wang et al., 2006). Additionally a role for $PGF_{2\alpha}$ and its FP receptor in endometrial adenocarinoma has been ascertained (Sales et al., 2005). An early experiment of phorbol ester (TPA) carcinogenesis in rat skin revealed that after TPA treatment a rapid increase in PGE_2 was observed followed by an increase in $PGF_{2\alpha}$ (Furstenberger et al., 1989). Tumour growth induced by TPA could be inhibited by indomethacin and rescued by the addition of $PGF_{2\alpha}$ but not PGE_2 to indomethecin treated tumours (Furstenberger et al., 1989). Furstenberger et al. suggested that PGE_2 was responsible for initial stage of carcinogenesis, involving hyperproliferation and $PGF_{2\alpha}$ upregulation, and subsequently $PGF_{2\alpha}$ was responsible for the later stage of carcinogenesis (Furstenberger et al., 1989). A more recent in vitro update of this experiment, utilising the COX-2 specific inhibitor NS398, found that $PGF_{2\alpha}$ co-treatment with TPA could rescue NS398 inhibited fibroblast cell transformation (Wolfle, 2003). Similar to earlier studies, prostaglandin $F_{2\alpha}$ was unable to initiate cell transformation therefore it is primarily a promoter of cell transformation in multi-step carcinogenesis (Wolfle, 2003). Previous research published by Sales et al. showed $PGF_{2\alpha}$-FP signalling in endometrial adenocarcinoma stimulates the production of growth factors (Sales et al., 2007; Sales et al., 2005). Similarly, PGE_2 signalling through the EP2 receptor can stimulate the secretion of growth factors in endometrial adenocarcinoma (Sales et al., 2004b). Recently, Rao et al. published an article showing that PGE_2 signals through the EP4 receptor to promote blood vessel growth in an in vivo sponge assay (Rao et al., 2007).

1.3.5 Prostaglandins as inflammatory mediators

Prostaglandins are lipid mediators of inflammation and their role in inflammation has been investigated over the last 30 years (Kundu and Surh, 2008). Both

prostaglandins and their catalytic COX enzymes are implicated in the mediation of inflammation within and out with the reproductive tract. As COX-2 is the inducible COX enzyme, which increases in prostaglandin production in response to external stimuli, it is this isoform that has been most widely studied in inflammation of malignant diseases (Kundu and Surh, 2008). Prostaglandin E_2 is the most thoroughly studied COX-2 product in inflammation (Kundu and Surh, 2008). The expression of COX-2, along with prostaglandin production, is elevated in inflammatory disorders including intestinal cancer, rheumatoid arthritis and endometriosis (Kundu and Surh, 2008). COX-2 and PGE_2 expression are induced by inflammatory cytokines including CXCL1 and CXCL8 in cancer, as well as bacterial lipopolysaccharide (LPS) in an inflammatory disease of the bovine endometrium endometritis, and phorbol esters (Furstenberger et al., 1989; Herath et al., 2006; Kundu and Surh, 2008). mPGES expression is induced by cytokine IL-1β or LPS treatment in a variety of cell types (Murakami et al., 2002). Both prostaglandin E_2 and COX-2 can promote cell proliferation and prevent apoptosis (Jain et al., 2008; Krysan et al., 2005; Sales et al., 2004c; Tsujii and DuBois, 1995). In vivo, PGE_2 and COX-2 promote tumorigenesis in experimental animals (Amano et al., 2003; Jain et al., 2008; Rao et al., 2007). Additionally, prostaglandins including PGE_2 and $PGF_{2\alpha}$ can act as an immune cell chemoattractants (Arnould et al., 2001; Kundu and Surh, 2008). In malignant oesophageal cells, Abdalla et al. showed that inflammation was not necessarily the main cause of COX-2 upregulation but chronic inflammation and COX-2 expression in combination contribute to tumour progression (Abdalla et al., 2005). Prostaglandins can upregulate many inflammatory mediators including CXCL1 and CXCL8 as well as COX-2 (Sales, 2009; Wallace et al., 2009; Wang et al., 2006). Therefore, in inflammatory diseases COX-2 and prostaglandins may help to maintain a persistent inflammatory state through a positive feedback loop. This may be one mechanism supporting the theory that tumours are wounds that do not heal (Dvorak, 1986).

1.3.6 Prostaglandins in endometrial adenocarcinoma

The secretion of prostaglandin E_2 is elevated in endometrial adenocarcinoma samples compared to normal endometrial samples (Jabbour et al., 2001). In addition, the levels of PGE_2 receptors, EP2 and EP4 are also upregulated in endometrial adenocarcinomas samples compared to normal endometrial samples (Jabbour et al., 2001; Sales et al., 2004b). Jabbour et al. found that COX-2, PGES, PGE_2 and EP4 were expressed in the glandular epithelial and endothelial cells of well, moderately and poorly differentiated endometrial adenocarcinomas (Jabbour et al., 2001). Research by Sales et al. showed that PGE_2 treatment of endometrial adenocarcinoma explants increased VEGF-A secretion (Sales et al., 2004b). Both the FP receptor and VEGFA mRNA are upregulated in endometrial adenocarcinoma (Sales et al., 2005). In endometrial adenocarcinoma samples, the FP receptor colocalises with VEGF-A protein and the endothelial cell marker CD31 (Sales et al., 2005). The expression of prostaglandins and their receptors on the vasculature of endometrial adenocarcinoma suggests a direct role for prostaglandin E_2 and $F_{2\alpha}$ in the regulation of endometrial adenocarcinoma blood vessels. In addition, the expression of angiogenic proteins induced by prostaglandins suggests that PGs may control the paracrine action of growth factors on the vasculature.

Both $PGF_{2\alpha}$ and PGE_2 can directly stimulate the proliferation of endometrial glandular epithelial Ishikawa cells (Milne et al., 2001; Sales et al., 2004c). Furthermore, the expression of the potent growth factor FGF2 is also upregulated in endometrial adenocarcinoma samples compared to normal endometrial samples (Sales et al., 2007). FGF2 expression is induced by both $PGF_{2\alpha}$ and prostacyclin in Ishikawa cell lines (Sales et al., 2007; Smith et al., 2006). Sales et al. found that FGF2 contributed to the autocrine regulation of Ishikawa cell proliferation by $PGF_{2\alpha}$. In addition, Furstenberger et al. found that PGE_2 and $PGF_{2\alpha}$ promote cell transformation (Furstenberger et al., 1989). It is therefore possible that hyperproliferation of endometrial cells, required for progression to a malignant state, could be enhanced by the autocrine/paracrine effects of prostaglandin-receptor signalling.

PGs may be involved in the mechanisms controlling cell metastasis and invasion (Sales et al., 2008). Recently, Sales et al. found using Ishikawa cells stably overexpressing the FP receptor (FPS cells), that prostaglandin $F_{2\alpha}$ signalling through the FP receptor stimulated changes in lamellipodia formation, as a result of actin cytoskeletal reorganisation, and enhanced cell migration (Sales et al., 2008). In addition, this alteration in cell morphology enhanced cell integrin contacts with the extracellular matrix and led to an increase in cell adhesion (Sales et al., 2008). The cellular processes of adhesion and migration are important factors in the invasion and metastatic spread of cancers (Avraamides et al., 2008; Bergers et al., 2003).

As mentioned in the previous section, prostaglandins are inflammatory mediators and their role as such may influence the initiation and progression of endometrial adenocarcinoma (Kundu and Surh, 2008). Consequently, the evidence from previous research suggests that in endometrial adenocarcinoma, prostaglandins may be involved in vascular formation, cell proliferation, cell transformation, cell migration and inflammation, thereby contributing to tumour progression.

1.4 Prostaglandin signalling in endometrial adenocarcinoma.

Prostaglandins increase the transcription of proteins through signalling pathways, the contents of which depend on the specific prostaglandin and receptor interacting. As GPCRs, the downstream signalling of the prostaglandin receptor is in the first instance determined by the G-protein α subunit to which it is coupled. For example, the FP, EP1 and EP3 receptor couple to the Gαq (Gq) whereas EP2, EP3 and EP4 couple to Gαs (Gs). GPCRs do not form stable multiprotein complexes but instead activate enzymatic effectors that stimulate second messenger signalling pathways (Luttrell and Luttrell, 2004). Gq is responsible for activating IP_3 after binding and activation of phospholipase C (PLC). Alternatively the Gs can bind and activate adenylyl cyclase (AC) which subsequently activates cyclic AMP (cAMP). In Ishikawa cells stably overexpressing the FP receptor, $PGF_{2\alpha}$ interacting with the FP receptor stimulates the release of inositol 1, 4, 5-triphosphate (IP3) (Fig. 4). This is via the action of the Gq protein and phospholipase C (Jabbour et al., 2006b). The

activation of IP3 leads to c-Src activation, Epithelial Growth Factor receptor (EGFR) signalling and subsequent Ras-ERK1/2 pathway activation. Similarly PGE_2 binding to an EP receptor causes an increase downstream Ras-ERK1/2 pathway activation via the cAMP pathway. Sales et al. found that cyclic AMP (cAMP) levels were increased after PGE_2 treatment in Ishikawa cells stably expressing the EP2 receptor and cAMP accumulation was similar to that seen in endometrial adenocarcinoma explants treated with PGE_2 (Jabbour et al., 2001; Sales et al., 2004b).

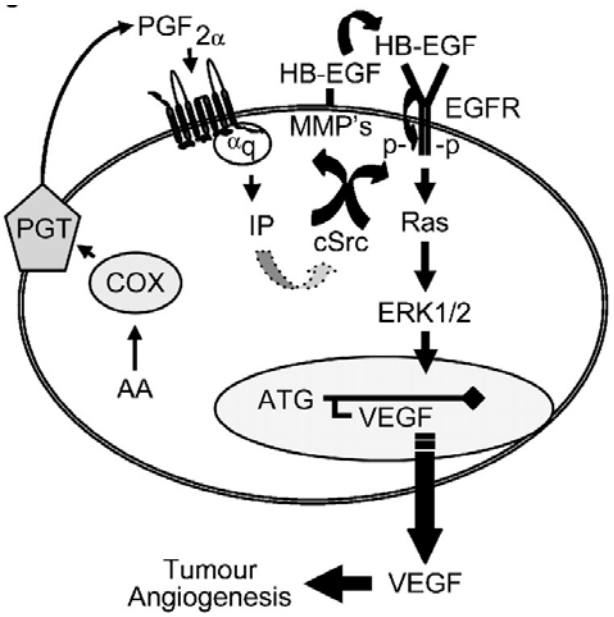


Fig. 4. $PGF_{2\alpha}$-FP signaling upregulates angiogenic VEGFA expression. Prostaglandin $F_{2\alpha}$, synthesized from AA by COX catalytic activity, is transported out of the cell by prostaglandin transporter (PGT). $PGF_{2\alpha}$ bind to Gq-coupled F-prostanoid receptor (FP) activating IP phosphorylation and cSRC. C-Src stimulates MMP cleavage of HB-EGF from the plasma membrane or directly phosphorylates EGFR. HB-EGF ligand activation of EGFR activates MAPKinase pathway involving Ras and ERK1/2 phosphorylation. Dimerised ERK1/2 can translocate to the nucleus and enhance VEGF transcription. VEGF secreted protein can act in a paracrine manner on endothelial cells to stimulate angiogenesis.

ERK1/2 activation can induce COX2 and subsequently gene transcription and the production of other factors occurs. COX2 has been shown to be upregulated in many cancers including colon cancer and endometrial adenocarcinoma (Sales et al., 2007) (Tsujii et al., 1998).

Prostaglandins, acting via their receptors, can increase the transcription and secretion of angiogenic factors in endometrial adenocarcinoma cells and consequently have the potential to stimulate angiogenesis (Sales and Jabbour, 2003). Previous studies have shown that treatment of FP-overexpressing Ishikawa cells (FPS cells) with $PGF_{2\alpha}$ promotes the release of angiogenic factors VEGF, bFGF, CXCL1 and CXCL8 (Sales et al., 2007; Sales et al., 2005; Sales, 2009; Wallace et al., 2009). These angiogenic factors may in turn function in an autocrine/paracrine manner on cells of the endometrium to promote angiogenesis.

In support of the involvement of the prostaglandin pathway in the mediation of cancer vascular remodelling, a recent study found that the coordination of COX-2, EGFR and MMPs are essential for progression of metastatic mammary tumours (Gupta et al., 2007). Gupta et al. demonstrated that combinational targeting COX-2, EGFR, MMP1 and MMP2 with shRNA significantly reduced mammary tumour xenograft vessel length, lumen number, vessel branching and vascular permeability and stated that it was this mode of action that was responsible for the reduction in pulmonary metastasis (Gupta et al., 2007). As described above, COX-2 activity catalyses the formation of prostaglandins which signal via the pathway described above. MMPs are a critical part of the transactivation of EGFR involved in the prostaglandin pathway as they cleave HB-EGF from the cell surface membrane allowing it to bind EGFR and activate downstream signalling such as the ERK1/2 pathway. In vitro studies using inhibitors of COX-2, EGFR, Src and MMPs abolish prostaglandin signalling (Battersby et al., 2007; Krysan et al., 2005; Sales et al., 2004a; Sales et al., 2005; Smith et al., 2006). Therefore, knocking out each of these components would perhaps multi-mechanistically apprehend prostaglandin receptor signalling and its effects on vascular function. It would be interesting to compare the efficacy of this combinational targeting with the targeting of individual prostaglandin receptors.

In addition to prostaglandin $F_{2\alpha}$-FP signalling via ERK1/2 in glioma cells, Ras/Raf activation can trigger Tcf transcription of immediate early response gene Cyr61 (Xu

et al., 2009b). Cyr61 is involved in angiogenesis and has been found to be upregulated in endometrial adenocarcinoma (Watari et al., 2009). Therefore, the upregulation of Cyr61 by $PGF_{2\alpha}$ in endometrial adenocarcinoma could be involved in the progression of the disease through mechanisms such as the regulation of angiogenesis.

1.5 Initiation and progression of cancer

To understand the factors affecting the initiation and progression of endometrial cancer it is important to be aware of the key processes of tumourigenesis. There are multiple genetic changes that can lead to the transformation of a normal cell into a cancer cell, thus factors regulating the initiation and progression of cancers vary depending on the cancer type. However, despite their differences, the progression of most invasive cancers can be defined by general steps, the hallmarks of cancer (Hanahan and Weinberg, 2000). These hallmarks, reviewed by Hanahan et al., obtained by cells in order to become fully tumourigenic are: insensitivity to anti-growth signals, self-sufficiency in growth signals, evading apoptosis/limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis, depicted schematically in Fig. 5 (Hanahan and Weinberg, 2000). In addition, the progression of cancer requires a resistance to hypoxia and acid-mediated toxicity as well as the assimilation of inflammation (Colotta et al., 2009; Gatenby and Gillies, 2008). Cell-cell competition governed by Darwinian rules, adaptive survival, is also proposed to apply in the context of cancer (Moreno, 2008).

Cell transformation from normal non-malignant to malignant cells involves somatic cell mutation. Numerous genes have been identified to be mutated in cancer thereby conferring oncogenicity. Among these are cell cycle regulators such as TP53, BCL-2 and MYC (Gatenby and Gillies, 2008). However, there are epigenetic influences such as DNA methylation and acetylation that control gene expression and cell transformation (Wu et al., 2005). Epigenetic changes have been found in endometrial cancer. For example, in a study by Wu et al., 75% of endometrial cancers were found to lack methylation of the Pax2 gene (Wu et al., 2005). Pax2 is expressed

during development but silenced by methylation in most adult cells (Shang, 2006). In endometrial cancer cell xenografts, Pax2 expression promotes tumour growth (see section 1.2.3 for more details) (Wu et al., 2005).

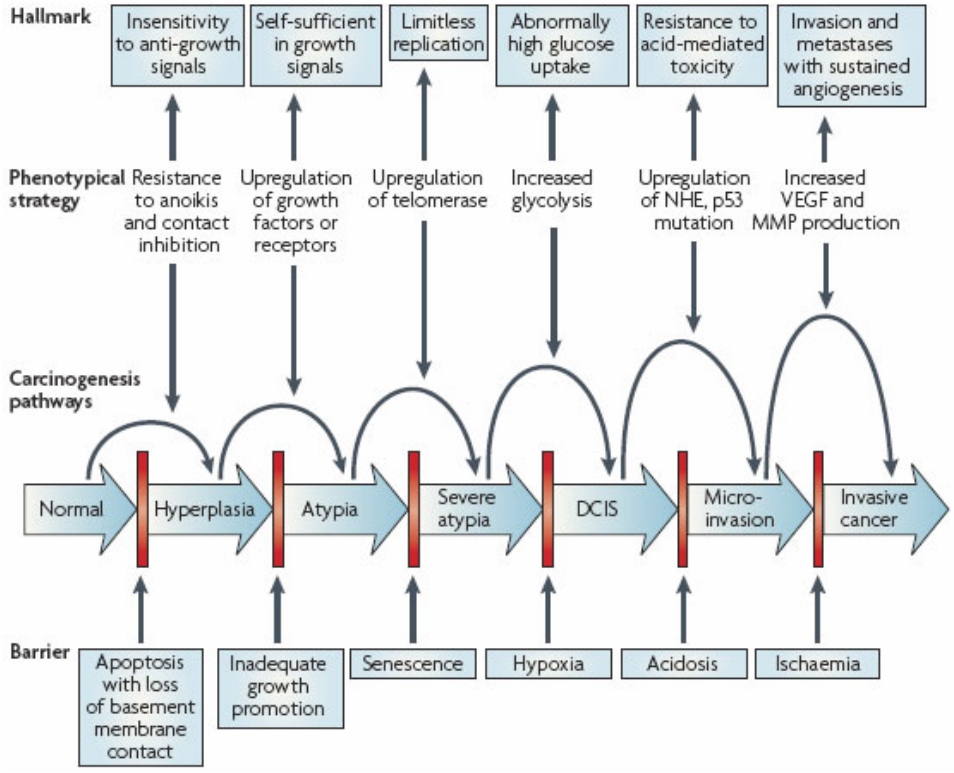


Fig. 5. The carcinogenesis pathway. Adapted from Gatenby et al. (2008). Carcinogenesis is the multi-step transformation from normal cells to hyperplasia, atypia (cells show abnormalities of the nucleus), ductal cancer in situ (non-invasive), microinvasive cancer ending in invasive and metastatic cancer. The microenvironmental barriers to proliferation influence the phenotypical strategy of cancer cells and the hallmarks of cancer define each step of the progression.

1.5.1 Microenvironmental barriers to proliferation.

Microenvironmental proliferative barriers shape tumour populations through selective adaptation, see Fig. 5 (Gatenby and Gillies, 2008). This is a theory described by Gatenby and Gillies in a recent review (Gatenby and Gillies, 2008). They hypothesise that the first genetic changes in tumourigenesis must enable the clonal expansion of dysplastic cells in situ and therefore involve either a mechanism to overcome contact inhibition or detachment-induced apoptosis, that is, anoikis

(Gatenby and Gillies, 2008). The initial proliferation of dysplastic epithelial cells in an avascular landscape, away from a blood supply and the growth factor gradient, requires cells to increase their autocrine-paracrine production of factors, upregulate cell surface receptors or increase transduction pathways (Gatenby and Gillies, 2008). An increase in signal transduction pathways regulating glucose metabolism helps tumour cells to survive in increasingly hypoxic conditions. Eventually however, the need for more oxygen and nutrients promotes in situ tumours to become invasive. Microinvasion, the migration of dysplastic cells into adjacent areas of normal cells, enables tumour cells to escape the constraints of cell-cell competition for substrates that limits their proliferation (Gatenby and Gillies, 2008). Tumour cells come into direct contact with blood vessels further advancing their proliferation. Consequently, Gatenby et al. propose that microinvasion is the critical adaptive landscape for tumourigenesis that involves the coordination and cooperation of tumour cells and neighbouring stromal cells (Gatenby and Gillies, 2008).

1.5.2 Co-ordination of different cell types

The progression of cancer requires the collaboration of many different cell types. Stromal cells, including immune cells, endothelial cells and fibroblast cells have been found to be essential for tumourigenesis in part due to the paracrine action of their secretory factors such as cytokines and growth factors (Hanahan and Weinberg, 2000). Bone marrow-derived myeloid cells, such as macrophages, neutrophils, eosinophils, mast cells and dendritic cells have all been shown to have a role in controlling vascular growth in cancers (Murdoch et al., 2008). For example, tumour associated macrophages (TAMs), are attracted to tumours by tumour secretory factors such as vascular endothelial growth factor (VEGF) (Murdoch et al., 2008). TAMs secrete cytokines such as CCL5 and CCL2 which promote cancer progression. Also, TAMs secrete factors that promote tumour blood vessel growth such as VEGF and fibroblast growth factor 2 (FGF2) (Murdoch et al., 2008). Bingle et al. found that in vitro co-incubation of TAMs with breast cancer cells increased blood vessel sprouts from spheroids as well as VEGF-A expression, and that in vivo increased the number of vessels formed in tumour spheroids (Bingle et al., 2006).

Recently, $CD4^+$ T cells were found to enhance malignant mammary breast invasion by secreting IL-4 and inducing the secretion of macrophage epidermal growth factor (EGF) (McCarthy, 2009).

1.5.3 The invasive and metastatic phenotype.

The migration of tumour cells to secondary sites around the body is known as metastasis. Certain tissues of the body are more susceptible to formation of secondary tumours, irrespective of blood flow, and these sites are referred to as metastatic niches (Psaila and Lyden, 2009). Current theories of metastasis suggest that invasive malignant cells from the primary tumour site can migrate and settle in metastatic niches where they form micrometastases (Psaila and Lyden, 2009). Once activated by the angiogenic switch, these micrometastases form macrometastases (Gao et al., 2009). Common metastatic niches include the lung, liver and lymph nodes. Recent research by Gao et al. found that bone marrow-derived endothelial progenitor cells (EPCs) are necessary for the transition from micro- to macrometastases in the mouse lung (Gao et al., 2009).

1.5.4 Evasion of apoptosis

Approximately 60 billion new cells are generated by the average adult human body everyday (Cotter, 2009). To balance this proliferation, cell death, apoptosis, is required. The evasion of apoptosis is required for cell transformation (Cotter, 2009). In order to avoid cell death, tumour cells use a variety of methods such as the upregulation of anti-apoptotic factors or the mutation of survival factors. One of the first anti-apoptotic factors identified and investigated was B cell lymphoma gene 2 (BCL2) (Cotter, 2009). This anti-apoptotic factor was classified as an oncogene due to its ability to enable a cell to survive in uncharacteristic conditions. In addition, TP53, a tumour suppressor gene is mutated in most human cancers, including endometrial adenocarcinoma (Doll et al., 2008). In non-cancerous cells the TP53 protein product, p53, functions as a transcription factor promoting growth arrest, DNA repair and apoptosis through mechanisms including, the activation of apoptotic

factor BAX (Brosh and Rotter, 2009). Therefore, the loss of function mutation of TP53 enables cancer cells to evade cell death.

The cell cycle regulator and tumour-suppressor phosphatase with tensin homology (PTEN) is frequently found to have loss of function mutations in cancer (Cully et al., 2006). In particular, Type 1 endometrioid cancers frequently exhibit PTEN mutations resulting in decreased PTEN expression (Kong et al., 1997). PTEN is a negative regulator of PI3K signalling (see section 1.7.2) whose phosphatase action converts PIP_3 to PIP_2, thereby reversing the kinase action of PI3K, shown in Fig. 6. As PI3K is involved in promoting cell proliferation stimulated by growth factor-receptor binding, PTEN acts as an antiproliferative factor (Cully et al., 2006). In addition, PTEN can inhibit cell proliferation dependent on ERK1/2, as well as prevent cell spreading and migration (Cully et al., 2006). Daikoku et al. found that the conditional deletion of PTEN, in the endometrium of mice, rapidly induced the occurrence of endometrial cancer (Daikoku et al., 2008). This induction of endometrial cancer by PTEN deletion was associated with an increase in COX-2 expression and increase in Akt phosphorylation (Daikoku et al., 2008).

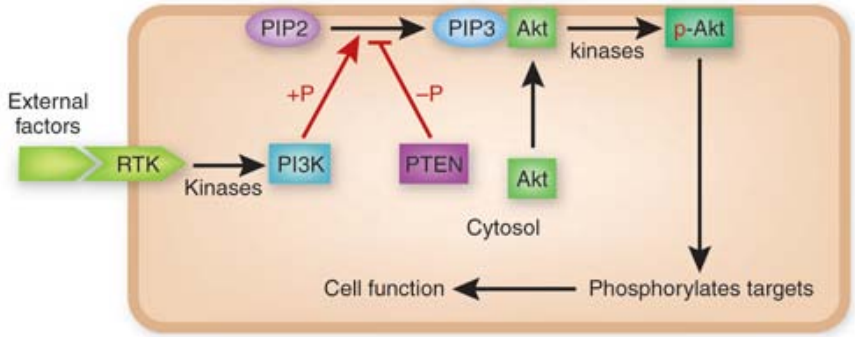


Fig. 6. PTEN regulation of the PI3K pathway. Adapted from Cully et al. (2006)

It is clear that cell transformation is essential for the transition from normal to cancerous cells however, this appears to be a two-way process. On rare occasions, cancer cells can revert back to normal cells (Telerman and Amson, 2009). For example, a survival factor, known as translationally controlled tumour protein (TCTP), is down regulated in cancer cells undergoing reversion. TCTP is a highly

conserved, ubiquitous, multifunctional, pro-survival factor that controls growth in mammalian embryonal stem cells, normal somatic cells and cancer cells (Telerman and Amson, 2009). Inhibition of TCTP in breast cancer cells can cause malignant cell growth to revert to normal cell growth (Telerman and Amson, 2009). Investigations into the mechanisms governing these rare events suggest that activating tumour reversion pathways could be a target for new treatments (Telerman and Amson, 2009).

1.6 Vascular function in cancer

Carmeliet recently stated that, 'Angiogenesis research will probably change the face of medicine in the next decades, with more that 500million people worldwide predicted to benefit from pro- or anti-angiogenesis treatments.' (Carmeliet, 2005). This concept has promoted interest in the field of angiogenesis research particularly in cancer.

1.6.1 Angiogenesis/neovascularisation

One process that is essential for the growth of any cancers larger than 2mm diameter is the formation of a sufficient blood supply (Abulafia et al., 1995). The blood supply delivers oxygen and nutrients to a tumour in order to meet its metabolic demand. As shown in Fig. 7, this formation of a new vasculature from existing vessels involves the coordination of many different cell types and molecular effectors including growth factors and basement membrane proteins. Tumour vascularisation is different from vascularisation in normal tissue. The tumour blood vessels formed are often irregularly shaped, dilated and can have dead ends (Bergers and Benjamin, 2003).

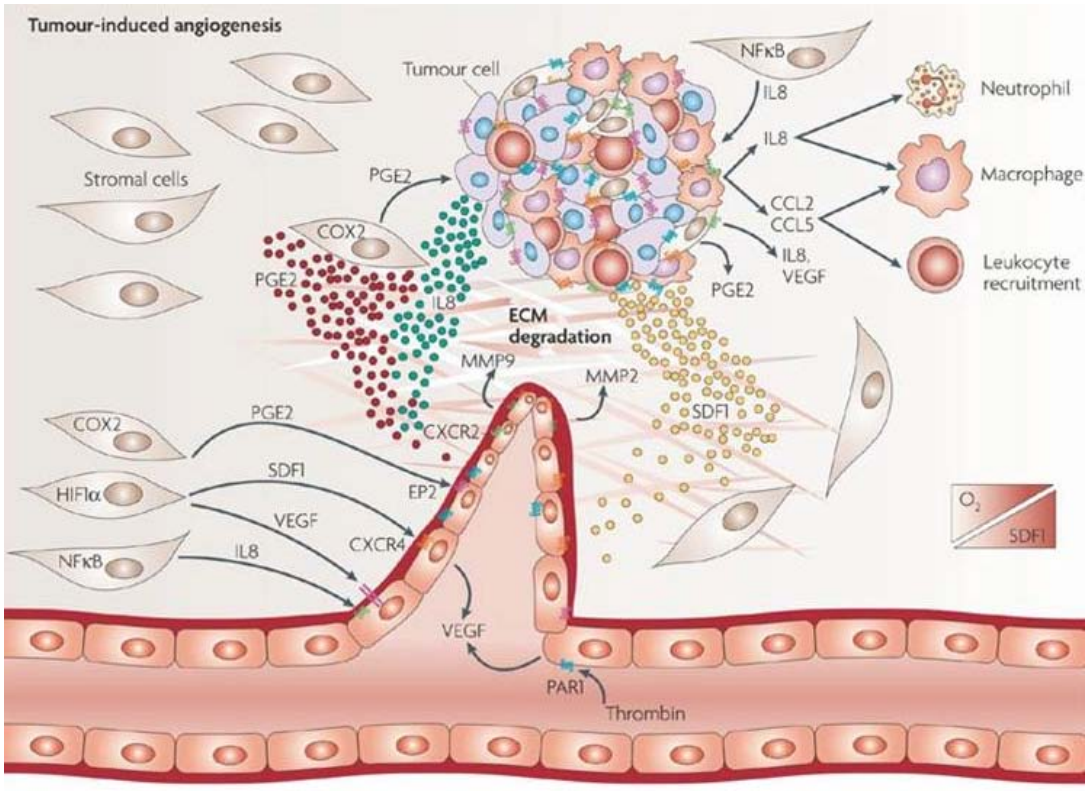


Fig. 7 Tumour-induced angiogenesis. Adapted from Dorsam et al. (2007).

As research has increased into the mechanisms of vascularisation a number of theories have arisen. The best know mechanism of angiogenesis, endothelial cell sprouting also called branching, was described by Auspunk and Folkman in 1977 (Fig. 7) (Ausprunk and Folkman, 1977). Sprouting angiogenesis is found in tumours and other sites of new vessel growth such as the corpus luteum before ovulation (Gargett and Rogers, 2001). This mechanism involves the degradation of the basement membrane surrounding a vessel, followed by the migration of the endothelial cells towards a chemotactic stimulus. The lumen of the new vessel is created by one or more endothelial cells curving whilst simultaneously being stabilised by reformation of the basement membrane and recruited pericytes (Bergers and Benjamin, 2003). Each step involves the coordination of different angiogenic factors (Ausprunk and Folkman, 1977).

In the vascular system the sprouting of endothelial cells to form vessel branches can be under the regulation of vascular endothelial growth factor-A (VEGF-A), Delta like 4 (Dll4) and the Notch pathway, shown in Fig. 8 (Horowitz and Simons, 2008). VEGF-A stimulates the secretion of Notch1 ligand Dll4 from endothelial tip cells. Dll4 then activates Notch1 which negatively regulates VEGFR2 expression in the cells behind the tip cells (phalanx cells) (Hellstrom et al., 2007). Thus a negative feedback loop prevents aberrant endothelial cell sprouting (Horowitz and Simons, 2008). This process is likely to be complemented by other signalling mechanisms for example, the matrix metalloproteinase MT1-MMP is also crucial for endothelial tip cell function and matrix invasion of developing branches (Stratman et al., 2009).

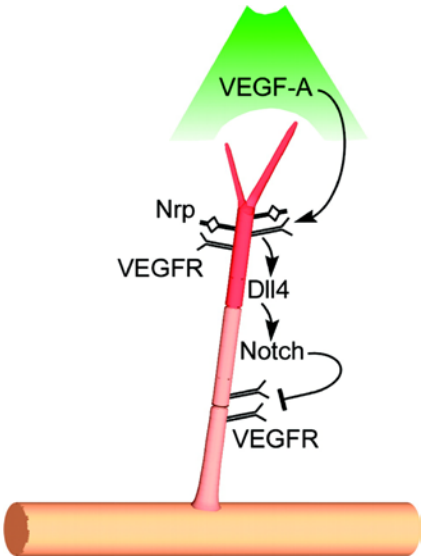


Fig. 8. Branching morphogenesis of blood vessels under the control of VEGF-A-Notch1 pathway. Adapted from Horowitz et al. (2008).

The schematic in Fig. 9 shows other modes of vessel formation seen in tumours, as well as endothelial cell sprouting, including vessel co-option, intussusception, vasculogenesis, vascular mimicry and glomeruloid 'looping' angiogenesis (Dome et al., 2007). Vessel co-option is defined as the growth of tumours along the existing blood vessels (Adams and Alitalo, 2007). This may occur in the early stages of tumour microinvasion or metastatic growth and also in tumours growing in vessel rich regions such as the brain (Dome et al., 2007). It is thought that the use of antiangiogenic drugs in the treatment of brain cancers may promote tumour growth

by vessel cooption as well as restoring the blood brain barrier and inhibiting the distribution of chemotherapy to tumour cells (Claes et al., 2008). Therefore, the tumour context and method of vascularisation is particularly important when considering antiangiogenic treatments.

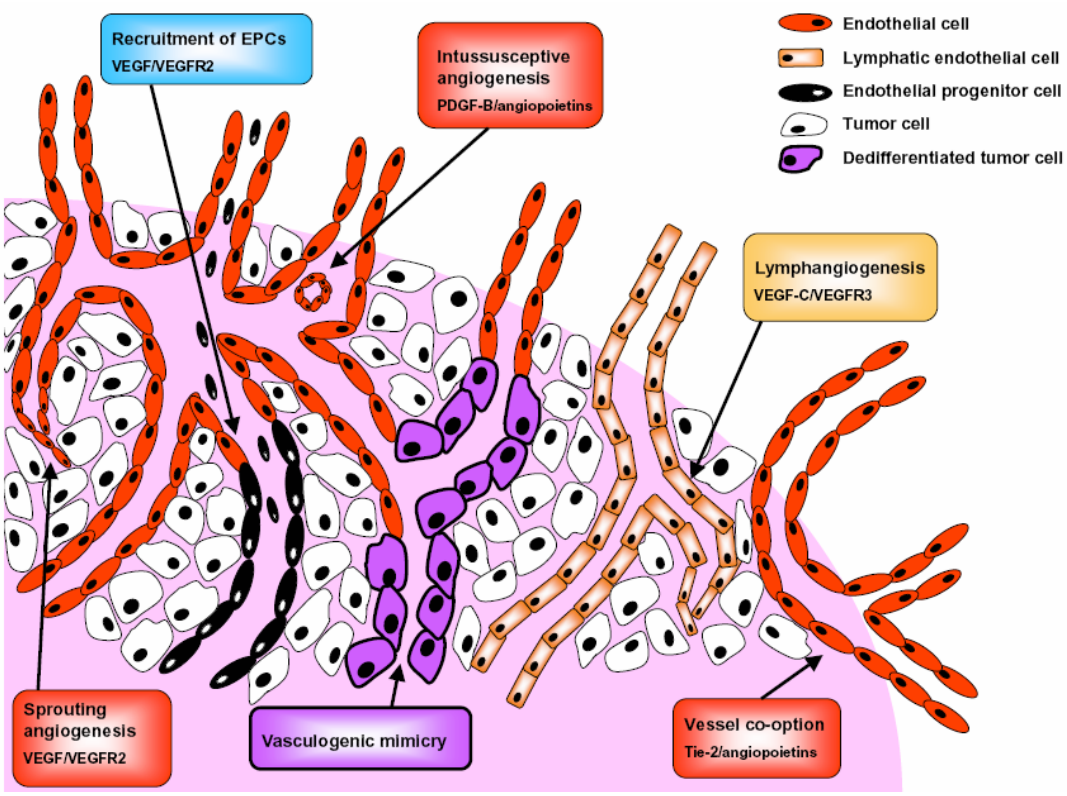


Fig. 9. Tumour vascularisation mechanisms. Adapted from Hillen et al. (2007).

Intussusceptive microvascular growth (IMG) is a process of vascularisation where existing vessels are divided by connective tissue thereby increasing the number of vessels (Burri and Djonov, 2002). This intussusception is seen in the developing lung but has also been investigated in the chick chorioallantoic membrane (CAM: see section 1.6.2.2) and cancer. In the CAM, vascularisation is initiated by angiogenesis followed by IGM to expand the vascular network (Burri and Djonov, 2002). A previous study found intussusception occured complementary to angiogenesis in wound healing (Kilarski et al., 2003). In large mammary tumours evidence of intussusceptive vascularisation is indicated by the presence of pillar and

mesh formations in a flattened vascular network as described by Djnov et al. in 2001 (Burri and Djonov, 2002).

Vasculogenesis, blood vessel formation de novo, is the development of new blood vessels from circulating endothelial progenitor cells (EPCs) originating from bone-marrow and EPCs in situ (Eguchi et al., 2007). This is an important process for the embryonic vascular development however its role in tumour angiogenesis is disputed. As mentioned previously, work has shown that bone marrow derived EPCs do not play a role in the formation of vessels in tumour vascularisation however; previous research has found that EPCs promote macrometastatic tumour growth (Gao et al., 2009; Purhonen et al., 2008). These discrepancies are likely due to differences in tumour type, stage and methods used to identify vessel-incorporated, BM-derived endothelial cells (Gao et al., 2009). Stem cell models, either 2D or 3D, such as embryoid bodies (EB) can be used to assess molecules regulating vasculogenesis from embryonic stem cells (Jakobsson et al., 2007).

Vascular mimicry also called ‘vasculogenic mimicry’ (VM) was a term used by Manoitis et al. to describe the process where cancer cells express an endothelial cell (EC) phenotype and form vessel-like networks (Dome et al., 2007; Hendrix et al., 2003). It is thought that tumour cells can redifferentiate into endothelial like cells and take on their roles to aid tumour growth (Hillen and Griffioen, 2007). Manoitis et al. found, using primary human uveal melanoma sections, that in many areas of the cancers the cells lining the blood vessels were not immunoreactive for endothelial cell markers, CD31, CD34 or von Willebrand factor (VWF) but were positive for S-100 protein, a characteristic of melanoma cells (Maniotis et al., 1999). VM melanoma networks were found to be unaffected by angiogenic inhibitors such as endostatin therefore the molecules regulating VM, such as EphA2, may differ from those regulating angiogenesis (Hendrix et al., 2003). Thus, once again it is important to identify the mode of vascularisation utilised by the tumour in order to tailor therapeutic treatments for optimum efficacy.

The term glomeruloid angiogenesis defines the process whereby tumour cells draw capillaries inward resulting in increased vessel loops (Dome et al., 2007). Recently, Kilarski et al. found, in a study of in vivo wound healing and angiogenesis of the CAM, that tension mediates rapid expansion of the vasculature by the elongation of vessel loops (Kilarski et al., 2009). This expansion of translocated vessels by biomechanical forces can also be called 'looping vascularisation' (Benest and Augustin, 2009).

Throughout the body there is another network of vessels called lymph vessels that maintain normal tissue homeostasis (Adams and Alitalo, 2007). Fluids that leak out of blood vessels and into the tissue, due to intravascular pressure, are collected by the lymph vessels and recycled back into the blood (Adams and Alitalo, 2007). In addition, the lymph vessels play an essential role in the regulation of the immune system by transporting antigen presenting cells and lymphocytes to the lymphoid organs (Rogers et al., 2008). Interestingly blood vessels developed first in evolutionary terms as lymph vessels are only found in amphibians and higher organisms (Adams and Alitalo, 2007). The lymph vessels are lined with lymphatic endothelial cells identified by their expression of lymph specific markers such as lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1), podoplanin and prox-1 (Rogers et al., 2008). The growth of lymph vessels also occurs in cancer along side angiogenesis (Adams and Alitalo, 2007). The mechanisms promoting lymph vessel growth are thought to be similar to those promoting blood vessel growth, mainly lymphangiogenesis in tumours (Avraamides et al., 2008). In endometrial adenocarcinoma peri-tumoural lymphatic endothelial vessels are present in larger amounts than in normal endometrial tissue, correlating with VEGF-C and -D expression, and this is may contribute to tumour progression and metastasis (Donoghue et al., 2007).

Although all these methods have been found to facilitate tumour growth, it is generally thought that angiogenic sprouting is the rate limiting vascular step involved

in the growth of most tumours (Horowitz and Simons, 2008). Thus, this thesis will focus on vascularisation by angiogenesis.

1.6.2 Vascular research in vitro and in vivo

1.6.2.1 In vitro

Organ culture assays, such as the rat aortic ring assay, are a commonly used in vitro method for the assessment of angiogenesis (Auerbach et al., 2003). For example, isolated rat aorta can be cut into segments and cultured on an extracellular matrix such as matrigel. Over a period of 7-14 days, the effect of exogenous factors, including recombinant proteins and cell conditioned medium, on the outgrowth of endothelial cells can be assessed (Auerbach et al., 2003). An advantage of this assay is that surrounding non-endothelial cells, such as pericytes, are present to enhance endothelial outgrowths (Auerbach et al., 2003). However, tumour angiogenesis primarily involves microvascular cells rather than the aortic endothelial cells. In addition, the assay conditions must be optimised so that the angiogenic effects of the treatment on endothelial cells can be distinguished from the effects on non-endothelial cells.

Cell culture models can be used in order to examine the effect of particular molecules on individual in vivo processes involved in angiogenesis. In vitro assays of endothelial cell migration, proliferation, differentiation (network formation), permeability and adhesion are used to identify the role of specific proteins and their receptors in the regulation of endothelial cell function (Auerbach et al., 2003; Folkman and Haudenschild, 1980). Advantages to in vitro cell culture are the ready availability of cells and avoiding the use of laboratory animals. Also assay conditions can be carefully monitored to limit variability and enhance reproducibility. However, cells in culture may express different proteins to those in vivo and the time period of investigations varies from in vivo situations therefore, in vitro results should be viewed with prudence and compared with in vivo data when possible (van Beijnum and Griffioen, 2005). Expression profiles of endothelial cells

in vitro has revealed that genes involved in endothelial cell proliferation of monolayers are cell cycle regulators whereas genes associated with network formation are involved in cell-cell/matrix contacts, cell matrix turnover and signalling (van Beijnum and Griffioen, 2005).

The number and scope of studies into endothelial cell biology increased after the isolation of endothelial cells from human umbilical cord veins by Nachman and Jaffe in the early 1970's (Nachman and Jaffe, 2004). Nachman and Jaffe used a set of criteria to confirm that the isolated cells were human umbilical vein endothelial cells (HUVECs) rather than fibroblasts (Nachman and Jaffe, 2004). These included: examining cell morphology as HUVECs form a monolayer of very flat, polygonal-shaped cells giving a cobblestone appearance, the presence of Weibel-Palade bodies in the cytoplasm, positive reactivity for ABO blood group antigens which was consistent with the blood type from the donor umbilical cord, and the expression of endothelial cell specific von Willebrand factor vIII (VWF, antihemophilic factor) antigen (Nachman and Jaffe, 2004). The in vitro culture of endothelial cells has enabled researchers to investigate the functions of endothelial cells in the normal vasculature as well as pathophysiological conditions such as cardiovascular disease and cancer. In the normal vascular system endothelial cells were identified as master regulators of blood fluidity due to their thromboregulatory role (Nachman and Jaffe, 2004). In cancer, the in vitro study of endothelial cells has identified angiogenic factors secreted by tumour cells that promote endothelial cell function (Folkman and Haudenschild, 1980; Folkman et al., 1971). This has led to the development of antiangiogenic drugs to target the formation and function of the tumour vasculature in order to disrupt tumour growth (Ferrara and Kerbel, 2005). Research in the past 30 years has continued to utilise HUVECs due to their ready availability, low cost, ease of isolation and identification.

1.6.2.2 In vivo

In vivo vascularisation models are used to verify the physiological relevance of the in vitro findings. Models include: the chick chorioallantoic membrane (CAM), the rat

cornea angiogenesis assay, the matrigel sponge mouse model and the tumour xenograft mouse model (Auerbach et al., 2003). The CAM is commonly used as an in vivo model to investigate tumour angiogenesis. The CAM is a double layer of mesoderm in which a rich vascular capillary network develops, connected to the embryonic circulation by arteries and veins (Ribatti and Kwang, 2008). The capillary plexus of the CAM mediates gas and nutrient exchange for the developing embryo. Vascularisation of the CAM is thought to occur initially by angiogenic sprouting followed by rapid vascular network expansion by IMG (Ribatti and Kwang, 2008). The coordination of endothelial cell functions during angiogenesis can be tested by use of agonists and inhibitors to target specific angiogenic pathways. In this way in vitro results can be verified. One advantage of the CAM is that up until day 10 the chick embryo is not immunocompetent so foreign cells grafted into the CAM are not rejected (Ribatti and Kwang, 2008). In the 1980's Palczak et al. used the CAM to graft endometrial adenocarcinoma samples and found that angiogenesis takes place during the growth of the grafted tumours (Palczak and Splawinski, 1989). Recently Scavelli et al. used the CAM to show that implanting sponges soaked in bone marrow myeloma endothelial cell conditioned medium promotes tumour vascularisation which can be inhibited by zoledronic acid, an inhibitor of the VEGF-A angiogenic response (Scavelli et al., 2007).

More recently, investigations into tumour vascularisation have favoured the use of assays including, the matrigel sponge mouse model and the tumour xenograft mouse model. These models allow the monitoring of tumour growth within a microenvironment more closely resembling the humans. The matrigel sponge mouse (or rat) model has been extensively used by researchers to assess the proangiogenic or antiangiogenic effect of soluble compounds or cells of interest in vivo (Akhtar et al., 2002; Auerbach et al., 2003; Kyriakides et al., 2001). The matrigel sponge mouse model involves for example, the injection of an angiogenic protein or cancer conditioned medium directly into subcutaneously implanted matrigel sponges. After 7-21 days of implantation and treatment in the host mice, the sponges are removed and vascular density can be assessed.

The tumour xenograft model involves injection of cells with tumourigenic potential, such as a cancer cell line, into mice with a compromised immune background to allow the formation of tumours in vivo (Curwen and Wedge, 2009). Immuno-compromised mice, such as nude mice lacking a thymus, are used so that the hosts do not reject the foreign cells (Rygaard and Povlsen, 1969). Commonly, the tumourigenic cells are injected subcutaneously into the flanks of mice because this allows the tumour growth to be non-invasively monitored visually and with bilateral calliper measurement throughout the course of an experiment. The tumours formed can be extracted from the mice and used to examine proliferation and vessel formation. A frequently used marker of proliferation is Ki-67 protein which is present in cell nuclei during all proliferative stages of the cell cycle but absent in resting cells (Gerdes et al., 1984).

1.6.2.3 Assessing microvascular density (MVD)

There are a number of endothelial cell specific markers including, CD31 (PECAM1), CD34, CD105 (endoglin) and vWF (Zhang et al., 2002). Immuno-staining of endothelial cell lined vessels with these markers enables the quantification of blood vessels, called microvascular density (MVD), within the tissue or tumour of interest. There are a variety of methods for the quantification of vessels such as the 'hotspot' method of counting, the Chalkey vessel count and more recent in vivo computational methods. The Chalkey vessel count can be performed on haematoxylin stained or endothelial marker stained sections of tissue. This assessment of angiogenesis has been used since the 1940's as a reproducible method of measuring microvascular density (MVD) (Chalkey, 1943).

In the context of tumour growth, MVD was approved by Weidner et al. for the assessment of breast cancer and prostate cancer (Weidner et al., 1993; Weidner et al., 1991). Weidner et al. associated a poorer prognosis and the progression of cancer with an increase in MVD (Weidner, 1993). This association together with the knowledge that tumour angiogenesis is essential for tumour growth led to an

increased interest in the possibility of reducing MVD, with antiangiogenic compounds, to prevent tumour growth. The method of MVD assessment used by Weidner et al. involved the identification of vessel dense areas (at high magnification), 'hotspots', in which the number of vessels was then counted per area (mm^2) (at low magnification) (Weidner et al., 1991). More recently, computational methods of MVD analysis have been performed such as the automated counting of vessels in tissue sections after the photographs of sections have been converted to bitmaps (Fox et al., 1995). Despite the advances in technology, the differences in accuracy of MVD assessment do not vary significantly with these ex vivo methods (Fox et al., 1995). However, in vivo, non-invasive, 3D-imaging of vessels in tumours with the use of fluorescent markers such as rhodamine-dextran, macromolecular contrast agents, PET scanning and magnetic resonance imaging are providing more informative, context dependent information about the 3D-structure of vessels (McDonald and Choyke, 2003). These methods can be used to quantify the number and spacing of blood vessels, measure blood flow and vascular permeability, and analyse cellular and molecular abnormalities in blood vessel walls (McDonald and Choyke, 2003).

Quantification of blood vessels is not by itself a measure of tumour angiogenic activity but it is a useful prognostic indicator in a wide variety of cancers (Hlatky et al., 2002). The minimum MVD relates to the metabolic burden set by the oxygen and nutrient consumption rate of the tumour, although MVD can exceed the metabolic burden. Similarly, MVD is not by itself a measure of tumour progression. In fact the tissues of some tumours do not appear to have an increase in MVD compared to the normal tissue, MVD can even be lower, possibly due to the fact that these tumours are better adapted to survive hypoxia (Hlatky et al., 2002). When assessing the effectiveness of antiangiogenic treatments, MVD should not be the only indicator as in some cases vessel number may decrease in proportion to tumour shrinkage so no overall decrease in MVD would be observed. Other markers such as circulating levels of VEGFR2+ve cells and EPCs should be used alongside MVD

counts, as well as in vivo vascular imaging techniques, to indicate the effectiveness of inhibiting angiogenesis (Hlatky et al., 2002).

1.6.3 Pro and Antiangiogenic factors regulating vascular function.

Increased microvessel density (MVD) and vascular proliferation in cancers, such as endometrial cancer, have been shown to be associated with poorer prognosis (Czekierdowski et al., 2008; Stefansson et al., 2006). In endometrial cancer there is an increase in microvessel density compared to normal endometrial control (Abulafia et al., 1995). Microvessel density is associated with disease progression as it is increased in higher grade (poorly differentiated) endometrial cancers compared to lower grades (well differentiated) (Abulafia et al., 1995). Each mode of vascularisation requires different stimulatory factors which may be secreted in part by the tumour cells. The expression and secretion of protein from endometrial adenocarcinoma differs considerably from that of normal endometrial cells. Many of the proteins, differentially expressed by endometrial adenocarcinoma, are involved in vascular formation and angiogenesis.

This thesis will focus on the angiogenic factors, secreted from endometrial cancer cells as a result of the $PGF_{2\alpha}$-FP receptor signalling, and their paracrine action on endothelial cells. Therefore a more detailed understanding of certain signalling molecules involved in the growth factor pathways in endothelial cells is required and described below.

1.6.4 Vascular endothelial growth factor-A (VEGF-A)

The most notorious angiogenic factor, vascular endothelial growth factor-A (VEGF-A), is expressed and secreted from endometrial adenocarcinoma in levels approximately 3-fold more than from normal endometrium (Sales et al., 2005). In addition to its secretion from glandular epithelial cells, VEGF-A secreted from neutrophils may regulate both normal endometrial and endometrial adenocarcinoma angiogenesis (Gargett et al., 2001). In the normal endometrium, the precise localisation and timing of VEGF-A expression is uncertain due to some conflicting

results (Krikun et al., 2004) but the presence of VEGF-A in the endometrium suggests that it will have a role in endometrial angiogenesis. In the endometrium, VEGF-A can be secreted apically by glandular epithelial cells (Hornung et al., 1998) and from the stroma during the proliferative phase of normal endometrial cycle (Gargett et al., 1999). This coordinates with an increase in VEGFR2 expression in capillaries of the proliferative phase (Meduri et al., 2000). A more recent study found that VEGF-A protein was detected only in endothelial cells of the early secretory phase of the endometrial cycle (Saito et al., 2007).

VEGF-A, also known as VEGF-165, belongs to a family of VEGF proteins consisting of four other members, VEGF-B, VEGF-C (VEGF-121), VEGF-D and placental growth factor (PlGF) (Ferrara et al., 2003). Together with co-receptors such as HSPGs, the VEGF proteins bind tyrosine kinase VEGF receptors 1, 2 and 3 (VEGFR1,2 and 3) (Ferrara et al., 2003). VEGFR1 and VEGFR2 bind to all the VEGF proteins whereas VEGFR3 binds only VEGF-C and VEGF-D. VEGFR1 and VEGFR2 are both commonly expressed on endothelial cells but VEGFR2 is the main receptor for transmitting VEGF-A intracellular signalling (Ellis and Hicklin, 2008). Studies to differentiate between the roles of VEGF-A to VEGFR1 or VEGFR2 signalling highlighted, using the rat corneal angiogenesis assay, that VEGFR2 but not VEGFR1 is required for angiogenesis in vivo (Gille et al., 2001). Similarly, in guinea pig skin, the Miles assay showed that VEGFR2, but not VEGFR1, was essential for increasing vascular permeability (Gille et al., 2001). In contrast, VEGFR1 was responsible for mediating VEGF-A induced migration of porcine aortic endothelial cells (Gille et al., 2001). The same group also showed in HUVECs that VEGFR2, but not VEGFR1, is responsible for downstream activation of phosphoinositide 3-kinase (PI3K), p38 mitogen-activated protein kinase (P38), extracellular signal-regulated kinase 1/2 (ERK1/2) and phospholipase C (PLC) (Gille et al., 2001). As shown in Fig. 10, VEGFR2 is a tyrosine kinase receptor and after autophosphorylation of Tyr1054 and Tyr1059, can bind to PLCγ and Shb domains in receptor substrate proteins (Fig. 10) (Olsson et al., 2006). An important in vivo mouse study demonstrated that VEGFR2 phosphorylation at Tyr 1173 in the PLC

binding site is essential for vascular function in mouse embyrogenesis (Sakurai et al., 2005). In vitro, the activation of JNK and P38 pathways by VEGF-A has been shown to promote HUVEC tube formation and proliferation (Wu et al., 2006). Also, in human intestinal microvascular endothelial cells (HIMEC), the ERK1/2 pathway activated by VEGF-A treatment was essential for network formation and proliferation (Rafiee et al., 2004). In addition, VEGF-A to VEGFR2 signalling in HUVECs can increase the phosphorylation of focal adhesion kinase (FAK) to promote endothelial cell migration (Abedi and Zachary, 1997; Le Boeuf et al., 2006).

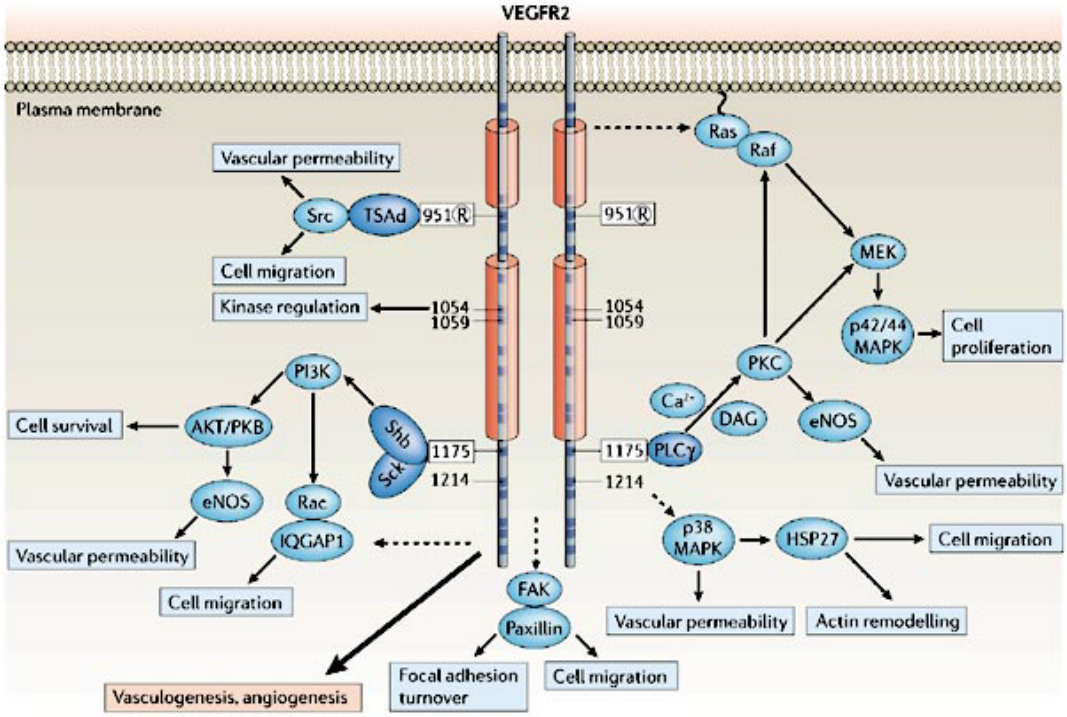


Fig. 10. Signaling intermediaries interacting with the intracellular domains of VEGFR2. Adapted from Ferrara et al. (2003).

VEGFR3 is expressed only on adult lymphatic endothelial cells however; it is widely expressed in developing endothelia (Tammela et al., 2008). Importantly, the expression of all VEGF receptors has been shown to be increased in a variety of tumours, both on epithelial and endothelial cells. Recently VEGFR3 has been identified as regulating vascular formation via endothelial sprouting, branching and

proliferation in mouse tumour xenografts, postnatal retina and embryonic development (Tammela et al., 2008). VEGFR3 is expressed in tip endothelial cells and research suggests that VEGFR3 expression can be induced by VEGF-A/VEGFR2 signalling and decreased by a regulator of angiogenic sprouting, Notch (Tammela et al., 2008). Upon ligand binding, the VEGF receptors can form homo- or heterodimers, perhaps explaining any over lap in receptor function.

The VEGFRs and in particular, VEGFR2 can coordinate with other growth factor receptors, GPCRs, integrins, neuropilins and cadherins to facilitate downstream signalling (Tammela et al., 2005). For example, transactivation of VEGFR2 by CXCR2, the GPCR for CXCL8 and CXCL1, is required to elicit CXCL8 induce endothelial cell permeability (Petreaca et al., 2007). In HUVECs, VEGF-A binding to VEGFR2 induces the internalisation of junctional protein VE-Cadherin as part of its role in vascular permeability (Gavard and Gutkind, 2006). Neuropilins can act as alternative receptors for VEGF-A and in this manner have been shown to enhance tumour angiogenesis (Geretti et al., 2008).

VEGF-A-VEGFR2 can activate integrin alphaVbeta5 which is dependent on Src and PKC activation to facilitate angiogenesis (Avraamides et al., 2008; Friedlander et al., 1995). In addition, integrin alphaVbeta3 is needed for maximal VEGFR2 activation and optimal HUVEC migration, polarisation and proliferation (Soldi et al., 1999). Integrin inhibitors have not been as effective as expected at inhibiting angiogenesis in various cancers during clinical trials (Reynolds et al., 2009). A recent research article demonstrates, in melanoma and lung carcinoma tumour grafts, that at low concentrations alphaVbeta3/beta5 integrin inhibitors actually promote tumour angiogenesis (Reynolds et al., 2009). Reynolds et al. show that VEGFA-VEGFR2 induced in vitro HUVEC network formation and migration can be enhanced by the addition of the alphaVbeta3/beta5 inhibitor which suppresses VEGFR2 degradation (Reynolds et al., 2009). This research highlights the importance of understanding the concentration dependent effects of potential inhibitors in vivo and in vitro.

In the normal physiological state, the role of autocrine endothelial VEGF is crucial for maintenance of vascular homeostasis (Lee et al., 2007). For example, Lee et al. showed in mice lacking endothelial VEGF expression, endothelial cell survival is reduced due to absence of intracellular VEGFR2 phosphorylation (Lee et al., 2007). This intracellular activation of VEGFR2 may be what distinguishes autocrine VEGF signalling from paracrine VEGF signalling. Their hypothesis that VEGF may act through internalised VEGFR2 receptors on endosomes, to initiate signalling pathways distinct from the traditional paracrine signalling pathway, is particularly interesting (Lee et al., 2007).

The cyclooxygenases and VEGF-A have been linked in pathways leading to increased angiogenesis in cancer. Both COX-2 and VEGF-A expression is increased in endometrial adenocarcinoma (Jabbour et al., 2001; Sales et al., 2005). VEGF-A to VEGFR2 downstream signalling can induce both COX-1 and COX-2 expression in HUVECs (Murphy and Fitzgerald, 2001). This results in the secretion of prostacyclin (Murphy and Fitzgerald, 2001). Similarly, in HUVECs VEGF-A can upregulate prostaglandin E_2 secretion by increasing COX-2 expression (Tamura et al., 2006). Alternatively, COX-2 upregulates VEGF-A expression to facilitate angiogenesis and tumour growth in a sponge implant model and tumour xenograft model (Yoshida et al., 2003).

Receptor tyrosine kinase inhibitors with selectivity for VEGFR, such as sorafenib and sunitinib are currently being used in clinical cancer trials (Ellis and Hicklin, 2008). Bevacizumab (Avastin) is a humanized monoclonal antibody against VEGF is approved for treatment of colorectal cancers, metatstatic breast cancer and non-small cell lung cancer (Ellis and Hicklin, 2008). However, combinational treatment of chemotherapy and radiotherapy appears to elicit a greater response than any individual treatment (Ellis and Hicklin, 2008). An in vivo investigation using an orthotopic mouse model of uterine carcinoma suggested that Bevacizumab treatment in combination with docetaxal, an inhibitor of cell division, may be effective at

reducing angiogenesis and tumour growth of endometrial adenocarcinoma (Kamat et al., 2007).

1.6.5 Angiopoietins

In addition to VEGF-A, Angiopoietin 1 (Ang1) and Angiopoietin 2 (Ang2) are potent angiogenic growth factors (Augustin et al., 2009). A study by Krikun found that Ang 1 mRNA was expressed mainly in human endometrial stromal cells (HESC) rather than glandular epithelial (HEGE) or endothelial (HEEC) cells (Krikun et al., 2004). Ang 2 mRNA expression was mainly in HEECs, followed by HEGEs and then minimal expression in HESCs (Krikun et al., 2004). Interestingly, the timing and localisation of Ang 1 and Ang2 expression may be related to their functions as Ang1 acts later in angiogenesis to stabilise vessels and recruit pericytes, and Ang 2, which can be upregulated by VEGF, acts earlier in angiogenesis to promote vascular remodelling and enhance vascular permeability (Krikun et al., 2004). In endometrial adenocarcinoma a study by Holland et al. found that there was minimal expression of Ang1 and high levels of Ang2 in poorly differentiated cancers (Holland et al., 2003). In contrast, a study by Saito et al. showed that Ang2 protein expression was lower in moderately and poorly differentiated endometrial adenocarcinoma than normal endometrium proliferative samples and there was no difference in the protein expression of Ang1 (Saito et al., 2007). It is possible that the variability of angiopoietin expression throughout the course of tumour progression make it hard to gain a clear assessment of its expression (Nasarre et al., 2009).

1.6.6 Tissue Factor

Tissue Factor (TF), also known as coagulation factor III and CD142, is a proangiogenic factor involved in both normal physiological and pathological angiogenesis although the mechanisms by which TF regulates these angiogenic states differs (Belting et al., 2004). TF, a transmembrane glycoprotein, is expressed at low levels in normal endometrial stroma but is absent from endothelial and glandular cells (Krikun et al., 2004). In contrast TF expression is high in endometrial adenocarcinoma and expressed by both epithelial and endothelial cells (Krikun et al.,

2004). TF has been associated with leaky vessels and proangiogenic thrombin production (Krikun et al., 2004). Thrombin G-protein coupled receptors known as protease activated receptors, PAR 1 and 2, can coordinated with TF to regulate angiogenesis in vivo (Belting et al., 2004). This suggests that in endometrial adenocarcinoma TF may be associated with increased angiogenesis and vascular permeability and thereby facilitating tumour cell growth and intra/extravasation.

1.6.7 Fibroblast growth factor 2 (FGF2)

Proangiogenic factor fibroblast growth factor 2 (FGF2) is one of the most potent promoters of epithelial growth and angiogenesis in vitro and in vivo (Presta et al., 2005). Research by Sales et al. has shown that FGF2 expression and secretion is upregulated in endometrial adenocarcinoma compared to normal endometrial samples (Sales et al., 2007). In addition, increased levels of FGF2 in malignant endometrial cancer correlate with the additional angiogenic growth factors IGF and EGF (Soufla et al., 2008). Soufla et al. found FGF2 mRNA overexpression in 75% of their endometrial cancer samples examined (Soufla et al., 2008). FGF2 and its receptor FGFR1 are expressed by both the glandular epithelial and vascular cells of endometrial adenocarcinoma (Sales et al., 2007). Sales et al. have shown that secretion of FGF2 stimulated by prostaglandin $F_{2\alpha}$ can promote the proliferation of endometrial adenocarcinoma cells via its FGFR1 receptor (Sales et al., 2007). The secretion of FGF2 from endometrial adenocarcinoma cells may also be regulated by the reproductive hormones oestrogen and progesterone (Fujimoto et al., 1997; Sales et al., 2007). Also, Pollock et al. identified eleven missense mutations at a number of locations of the FGFR2 receptor in endometrial cancer (Pollock et al., 2007). The most common mutation was S252W, which is also found in individuals with Apert syndrome and has been shown in vitro to enhance FGF ligand binding (Pollock et al., 2007). In endometrial adenocarcinoma cells lines FGFR2 inhibition in the context of common loss of function mutations such as PTEN result in cell death (Byron et al., 2008). These findings suggest that targeting the FGFR receptors could be a therapy for endometrial adenocarcinoma (Byron et al., 2008). However, the role of

endometrial adenocarcinoma derived FGF2 in the regulation of endometrial adenocarcinoma vascular function has yet to be investigated.

FGF2, also known as basic fibroblast growth factor, is one of 18 mammalian proteins belonging to the FGF family (FGF1-10 and FGF16-23) (Beenken and Mohammadi, 2009). FGF2 shares a homologous core region with the other FGFs but differs from them in the N- and C-terminal regions allowing for differential functional activity. In contrast to most of the FGFs, FGF2 is secreted through unconventional secretory processes involving export via a plama membrane resident transporter or shedding of plasma membrane vesicles (Walter Nickel, 2005). Once in the extracellular matrix, the FGF proteins signal through FGFRs to elicit a cellular response. Situated in the core region of the FGFs is a heparan sulphate proteoglycan (HSPG) (also known as heparan sulphate glycosaminoglycan HSGAG) binding site (HSB). HSPGs are located in the extracellular matrix near the surface of every cell. Binding of FGF2 to HSPG enhances its interaction with the FGFRs, hence enhancing FGFR activation and downstream signalling. There are four FGFR proteins (FGFR1-4) which each have various isoforms created by exon skipping and alternative splicing. Once FGF2 is bound, FGFRs dimerize enabling a series of auto-phosphorylation events resulting in activation of downstream signals. As shown in Fig. 11, FGFR downstream signalling is conveyed through two main interacting proteins, phospholipase C (PLC) and FGF receptor substrate 2 (FRS2) (Beenken and Mohammadi, 2009).

Endothelial cells most commonly express FGFR1(3c) and FGF2-FGFR1 signalling is implicated in the basal lamina degradation, migration, proliferation, morphogenesis and vessel maturation stages of angiogenesis (Auguste et al., 2003; Presta et al., 2005). Canonical FGF2-FGFR1 signalling in endothelial cells can induce many intracellular signal transduction pathways, including phosphoinositide 3-kinase (PI3K), p38 mitogen-activated protein kinase (P38), c-Jun NH(2)-terminal kinases (JNK), signal transducer and activator of transcription 3 (Stat3), mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK)1/2 pathways (Deo et al., 2002; Kanda et al., 1997; Matsumoto et al., 2002; Murakami et

al., 2008). Negative regulators of the FGF2-FGFR1 canonical signalling pathways include SEF, sprouty and MPK3 (Murakami et al., 2008).

Non-canonical signalling stimulated by FGF2 in endothelial cells involves the interaction of FGF2 with syndecans, integrins and neural cell adhesion molecules (NCAM), see Fig. 11 (Murakami et al., 2008). For example, syndecans, a member of the HSPG family, can act as FGF2 receptors (Murakami et al., 2008). Syndecans are abundant on the cell surface so despite their lower affinity for FGF2 than the FGFRs, they can significantly affect FGF2 signalling (Horowitz et al., 2002). In rat fat pad endothelial cells mutation of syndecan 4 obstructs FGF2 induced PIP_2 activation of PKC which disrupts endothelial cell proliferation, migration and network formation (Horowitz et al., 2002). Similarly, FGF2 can bind to vitronectin receptor alphaVbeta3 and mediate endothelial cell adhesion and spreading in vitro (Rusnati et al., 1997).

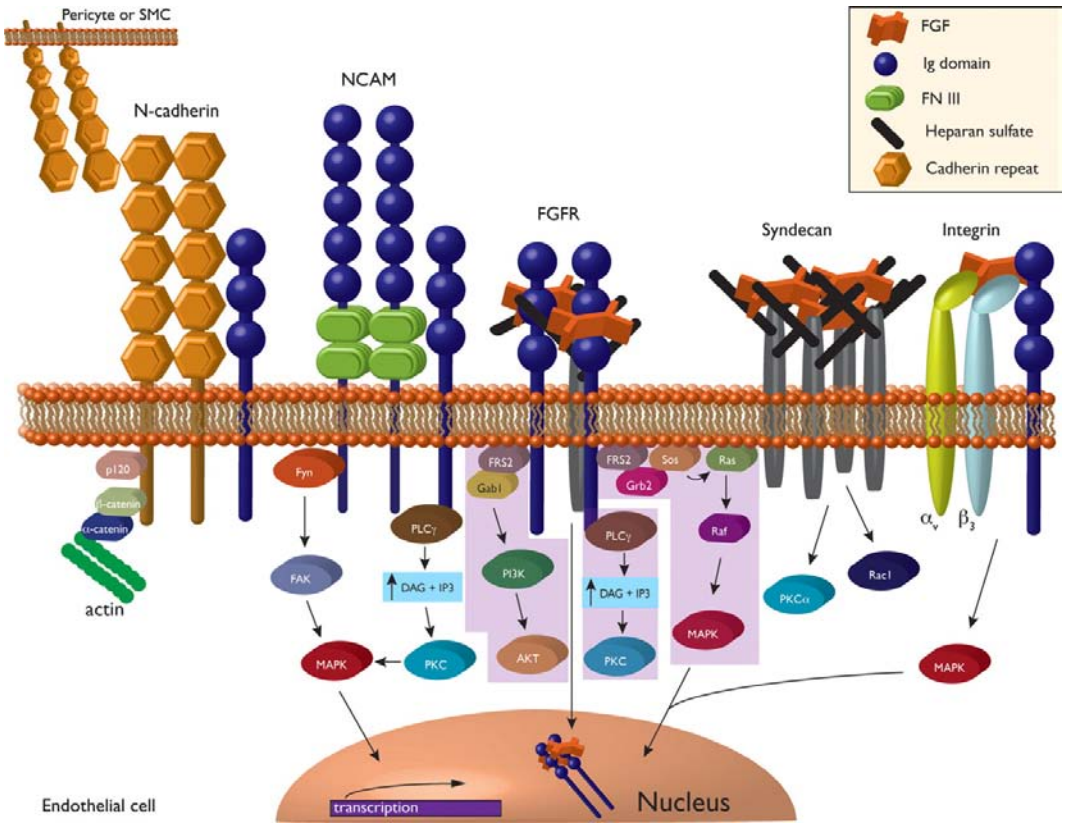


Fig. 11. Canonical and non-canonical FGF2 signalling pathways. Adapted from Murakami et al. (2008). FGF2 signals through FGFRs in the canonical pathways. FGFRs activate PI3K and MAPK pathways through interaction with receptor substrate proteins PLC

and FRS2. Non-canonical signaling involves FGF2 binding to alternative receptors such as syndecans and integrins.

Studies into anti-angiogenic treatments for cancer have investigated targeting FGF2-FGFR1 signalling (Beenken and Mohammadi, 2009). For example thalidomide is an FGF2 inhibitor as it increases the degradation of FGF2 mRNA and clinical trial in renal cancer patients suggested that it may increase stabilisation of the disease (Beenken and Mohammadi, 2009). More recent research has investigated the use of vesicles containing FGF2 in their outer lipid layer to target the delivery of drugs, such as thymidine kinase, to FGFR expressing cells in the tumour microenvironment (Beenken and Mohammadi, 2009).

1.6.8 Angiogenic Chemokines CXCL1 and CXCL8

Chemokines with an ELR motif Glu-Leu-Arg promote angiogenesis whereas those lacking the motif in general inhibit angiogenesis (Strieter et al., 2006). The ELR motif is a critical factor in determining the structure and hence receptor binding specificity for ELR chemokines. CXCL1, also known as growth related oncogene-α (GROα) or melanoma growth stimulatory activity (MSGA), and CXCL8, also known as Interleukin 8 (IL8), are both ELR containing angiogenic chemokines.

CXCL1 is a chemokine associated with inflammation, angiogenesis and tumourigenesis. Anisowicz et al. found GRO to be present normal human cells where its expression was low however, in human cancer cells its expression was increased (Anisowicz et al., 1987). In endometrial adenocarcinoma, CXCL1 expression is elevated compared to the normal endometrium (Wallace et al., 2009). The expression of CXCL1 by endometrial cancer cells may enhance neutrophil influx into endometrial adenocarcinoma (Wallace et al., 2009). Human endometrial stromal cells secrete CXCL1 (Nasu et al., 2001). Earlier research in 1989 identified that GRO was secreted by human umbilical vein endothelial cells (Wen et al., 1989). It is now established that in cancer the two main functions of CXCL1, secreted by tumour cells, endothelial cells, leukocytes and neutrophils, are to enhance immune cell tumour cell infiltration, through mechanisms involving chemotaxis and transendothelial cell migration, and promote angiogenesis (Kundu and Surh, 2008).

CXCL8 is an inflammatory chemokine and a proangiogenic factor (Li et al., 2003; Li et al., 2005). In the normal human endometrium, CXCL8 is expressed throughout the menstrual cycle with an increase in perivascular staining in the late secretory endometrium (Jones et al., 1997). This increase in CXCL8 expression coincided with leukocyte infiltration and COX-2 expression in premenstrual endometrium (Jones et al., 1997). In vitro data suggests that CXCL8 increases MMP 2 and 9 production in isolated, cultured stromal cells (HESCs) and treatment with CXCL8 enhances HESCs invasion of extracellular matrix (Mulayim et al., 2004). CXCL8 is also expressed in vascular and glandular cells of first trimester decidua along with prokineticin (Maldonado-Perez et al., 2009). Serum levels of CXCL8 are elevated in patients with endometrial cancer (Chopra et al., 1996). CXCL8 expression is increased in endometrial cancer, compared to normal endometrium, where it localises to the glandular epithelial and stromal cells (Sales, 2009). Maldonado-Perez et al. found that prokineticin treatment of endometrial cancer cells overexpressing the prokineticin receptor, increases CXCL8 production (Maldonado-Perez et al., 2009). CXCL8 mRNA and protein expression is elevated in endometrial cancer cells by nuclear activator of T cells (NFAT:see section 1.7.5) signalling (Sales, 2009). Alternatively, the expression of CXCL8 in endometrial microvascular endothelial cells can be regulated by lysophosphatidic acid in a p38-NFkB dependent mechanism to promote endothelial cell network formation and migration (Chen et al., 2008). In human endometrial endothelial cells (HEECs) from normal endometrium, the expression of CXCL8 is decreased by oestrogen and progesterone treatment (Luk et al., 2005). In contrast, CXCL8 expression is increased by oestrogen treatment in HEECs from women with endometriosis (Luk et al., 2005). These data, together with data showing the role of CXCL8 in angiogenesis, indicate that in an endometrial diseased state CXCL8 is an effector of vascular function under hormonal control. However, the role of CXCL8 on vascular function in the context of endometrial adenocarcinoma requires further investigation.

As chemokines, CXCL1 and CXCL8 modulate cellular functions by binding the chemokine receptors (CXCR). CXCL1 binds with high affinity to CXC receptor 2

(CXCR2) and with very low affinity to CXCR1 therefore CXCL1 is generally considered to be a CXCR2 specific ligand (Strieter et al., 2005). CXCL8 can bind effectively to CXCR1 and CXCR2 both of which are expressed on HUVECs (Li et al., 2003; Li et al., 2002). It is important to note that CXC receptor expression varies among different endothelial cell and human microvascular endothelial cells (HMECs) express higher levels of CXCR2 and are more responsive to CXCL8 than HUVECs (Salcedo et al., 2000). Both HMECs and HUVECs express CXCR3 and CXCR4 however neither CXCL8 nor CXCL1 act as ligands for either of these receptors (Salcedo et al., 2000). A recent article demonstrated, using a xenograft tumour model in mCXCR$^{-/-}$ and mCXCR2$^{+/-}$ mice, that levels of stromal CXCR2 are crucial for tumour angiogenesis and growth (Singh et al., 2009). Similarly, mCXCR-/- mice exhibit decreased corneal angiogenesis in response to CXCL8 (Addison et al., 2000). Also data by Keane et al. have demonstrated in mCXCR-/- mice a role for CXCR2 in tumorigenesis and angiogenesis in Lewis lung carcinoma (Keane et al., 2004a; Keane et al., 2004b). These data indicate that CXCL1 and CXCL8 signalling to CXCR2 on endothelial cells may be an important part of vascular formation in vivo (Singh et al., 2009).

The chemotactic effect of CXCL1 on immune cell migration has been widely studied in vitro but CXCL1 also plays a role in the regulation of endothelial cell function and angiogenesis (Bechara et al., 2007). In coronary artery disease, CXCL1 may inhibit endothelial vasorelaxation by decreasing the expression of endothelial nitric oxide synthase (eNOS) (Bechara et al., 2007). Recombinant CXCL1 can regulate bovine adrenal gland capillary endothelial cell migration and induce angiogenesis in vivo as shown using the rat corneal model (Strieter et al., 1995). In addition, recombinant CXCL1 induces endothelial cell proliferation and network formation and addition of CXCL1 antibody to HUVECs inhibits thrombin-induced endothelial cell proliferation and network formation (Caunt et al., 2006). In accordance, in the CAM angiogenesis assay, CXCL1 induces angiogenesis and thrombin induced angiogenesis is inhibited by addition of CXCL1 antibody (Caunt et al., 2006). Caunt et al. proposed a mechanism by which CXCL1 regulates angiogenesis by promoting

the expression of angiogenic factors, VEGF-A, VEGFR2, MMP9 and Ang2 (Caunt et al., 2006). Interestingly, CXCL1 expression was not upregulated by FGF2 or VEGF-A in HUVECs (Caunt et al., 2006). In a mouse xenograft model, tumour cells in which CXCL1 expression was knocked down with short hairpin RNA, exhibited decreased tumour xenogaft growth and microvessel density (Caunt et al., 2006). Similarly, in a human colorectal xenograft tumor mouse model, CXCL1-immunoneutralisation decreased tumour growth and angiogenesis (Wang et al., 2006). This was presumably because CXCL1 was required for cancer conditioned medium-induced effects on endothelial cell network formation and migration (Wang et al., 2006).

Paracrine and autocrine signalling of CXCL8 via the CXCR2 receptor has been shown to regulate endothelial cell network formation, proliferation and migration in vitro (Li et al., 2003; Li et al., 2005). Recombinant CXCL8 induces in vitro HUVEC proliferation and in vivo vessel growth in the rat corneal model of angiogenesis (Koch et al., 1992; Strieter et al., 1995). Most in vitro and in vivo data suggests that CXCR2 is the main CXCL8 receptor of endothelial cell function (Addison et al., 2000; Strieter et al., 2006). Addison et al. showed that CXCL8 induction of HMEC proliferation, migration and CAM angiogenesis was inhibited to basal levels by addition of a CXCR2 antibody (Addison et al., 2000). CXCL8 binding to CXCR2 stimulates signalling pathways downstream of this GPCR that regulate gene expression. For example, CXCL8 may promote the migration of HUVECs by increasing endothelial secretion of matrix metalloproteinases such as MMP9 and MMP2 (Li et al., 2003). Also, CXCL8 may regulate HUVEC survival by increasing the expression of antiapoptotic factor Bcl-xl and decreased the expression of antiapoptotic factor Bax (Li et al., 2003). In vitro and in vivo data by Yao et al. suggests that CXCL8 may direct angiogenesis via Sterol regulatory element-binding proteins (SREBPs) expression (Yao et al., 2006). SREBP is an important regulator of lipid membrane components and the activation of Rho GTPase, a protein involved in cytoskeletal remodelling, by SREBP was shown to be essential for CXCL8

induced endothelial cell network formation, proliferation, migration and in vivo CAM angiogenesis (Yao et al., 2006).

The differential roles of CXCL1 and CXCL8 have been shown to be due to the difference in their receptor binding affinity, as CXCL1 binds only CXCR2 whereas CXCL8 can bind CXCR1 or CXCR2. Interestingly, RhoGTPase was shown by Schraufstatter et al. to be downstream of CXCL8-CXCR1 activation regulating cytoskeletal arrangement (Schraufstatter et al., 2001). In contrast, CXCL8 or CXCL1 HMEC adhesion was found to be mediated by CXCR2 in a Rac dependent manner (Schraufstatter et al., 2001). However in vivo data suggests that CXCR2 is the main receptor mediating tumour progression as recent data by Warner et al. indicates that in head and neck tumours CXCL1 and CXCL8 secretion from endothelial cells enhances tumour cell invasion by signalling in a paracrine manner through CXCR2 on tumour cells (Warner et al., 2008).

As an alternative to directly stimulating intracellular signalling pathways, CXCR2 can transactivate the VEGFR2 and EGFR tyrosine kinases leading to downstream signalling from these receptors. In ovarian cancer cells, CXCL8 treatment induces phosphorylation of EGFR and MAPkinase pathway activation which are proposed to be responsible for alterations in cell migration (Venkatakrishnan et al., 2000). In endothelial cells, CXCR2, but not CXCR1, mediated HMEC migration is regulated by EGFR transactivation via cathespin and HB-EGF (Schraufstatter et al., 2003). Both CXCL1 and CXCL8 binding to CXCR2 can cause EGFR phosphorylation by HB-EGF which was also required for HMEC sprout formation in an in vitro model of angiogenesis (Schraufstatter et al., 2003). CXCL8 induced human microvascular endothelial cell permeability requires the transactivation of VEGFR2 (Petreaca et al., 2007). This coordination of CXCR2 and VEGFR2 signalling has recently been shown in murine endothelial cells whereby CXCR2 activation by CXCL8 increased VEGF-A expression, via a complex called CBM, and NFκB activation, hence, enhancing VEGFR2 activation (Martin et al., 2009). The effect of CXCL1-CXCR2 signalling on VEGFR2 transactivation has yet to be verified but is likely to be similar

to that induced by CXCL8 as they both activate CXCR2. A recent in vivo study suggests that inhibiting both CXCR2 and VEGFR2 may be an effective treatment to block pancreatic cancer growth (Li et al., 2009).

It is important to note that a study by Benelli et al. found that neutrophils are essential for CXCL1 and CXCL8 mediated angiogenesis as CXCL1 and CXCL8 failed to promote angiogenesis, measured by haemoglobin content in matrigel sponge implants, in mice lacking neutrophils (Benelli et al., 2001). This is interesting because in vitro CXCL1/CXCL8 and CXCR2 can directly control endothelial cell functions (Caunt et al., 2006; Li et al., 2005) and in the rat cornea, an environment free from immune cells, CXCL1 and CXCL8 induce angiogenesis (Strieter et al., 1995). In tumour angiogenesis the coordination of different cell types, i.e. endothelial and immune cells, is likely to be crucial.

A therapeutic antibody against CXCL8, ABX-IL8, has been developed and was shown to inhibit HUVEC network formation in vitro as well as inhibiting, in vivo melanoma tumour xenograft MMP production, invasion, growth and angiogenesis (Huang et al., 2002). However, the efficacy of ABX-IL8 in inhibiting cancer in clinical trials has not been investigated (Yan et al., 2006). Targeting the CXCL1/CXCL8 and CXCR2 receptors might not be an effective form of cancer therapy for all tumour types as a recent paper investigating the role of CXCR2 in cancer showed that CXCL8 to CXCR2 signalling can actually promote senescence thereby inhibiting growth of lung adenocarcinomas (Acosta et al., 2008). Clearly, more work needs to be done to elucidate the role of CXCL1/CXCL8 and CXCR2 in individual cancer strains.

1.6.9 The matrix metalloproteinases (MMPs)

Factors involved in the degradation of the extracellular matrix such as matrix metalloproteinases (MMP) 2 and 9 are also grouped with proangiogenic factors (Kalluri, 2003). The matrix metalloproteinases (MMPs) and Membrane-Type Matrix Metalloproteinases (MT-MMPs) are implicated in the regulation of angiogenesis,

invasion and metastasis of cancers (Davis and Senger, 2005; Egeblad and Werb, 2002; van Hinsbergh and Koolwijk, 2008). A recent microarray study by Du et al. found that MMP9 and MMP10 were upregulated in tumour associated endometrial endothelial cells (TEECs) compared to normal endometrial endothelial cells (HEECs) (Du et al., 2008). A study by Aglund et al. found that endometrial cancer patients with intense MMP9 and MMP2 immunohistochemical staining of tumours had a dramatically decreased survival rate than those patients with less intense staining for these MMPs (Aglund et al., 2004). Immunohistochemical analysis has also found that in endometrial cancer MMP9 expression is increased and can be correlated with increased COX2 and MMP2 expression, along with vascular and lymphatic invasion (Karahan et al., 2007). Park et al. suggest, using a 3D in vitro model of invasion with coculture of normal endometrial stromal cells and endometrial cancer cells, that MMP9 and MMP2 secreted by endometrial stromal cells, in an oestrogen regulated manner, aid endometrial cancer cell invasion (Park et al., 2001). In the normal endometrium, increased MMP9 expression has been associated with enhanced angiogenesis and vascular repair after menstruation (Plaisier et al., 2004). In vitro analysis of human microvascular endometrial endothelial cells (hMVEECs) found that they express MMP9 mRNA in response to various growth factor treatments including VEGF-A (Plaisier et al., 2004). Interestingly, in HUVECs, the secretion of MMPs was shown to be temporally regulated with a decrease in MMP9 and MMP2 production overtime that could be prevented by COX inhibition (Kiran et al., 2006). The secretion of MT3-MMP from endometrial endothelial cells was confirmed by Plaisier et al. using immunohistochemical analysis of endometrial tissue (Plaisier et al., 2004). hMVEEC network formation could be inhibited by MT3-MMP immunoneutralisation (Plaisier et al., 2004). These data suggest that MMPs, in particular MMP9, may be involved in regulation of angiogenesis and tumour invasion in endometrial adenocarcinoma.

1.6.10 Tissue inhibitors of metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs. For example, MMP9 activity is regulated by TIMP1, and MMP2 by TIMP2. Hence, TIMPS have been suggested to be antiangiogenic. In endometrial cancer a decrease in TIMP2 histological expression has been found to be associated with an increase in MMP2 expression and a poorer prognosis for endometrial cancer patients (Graesslin et al., 2006). Plaisier et al. found that adenoviral overexpression of TIMP1 and TIMP3 inhibits VEGF-A induced hMVEEC network formation in vitro (Plaisier et al., 2004). Both TIMP1 and TIMP2 can inhibit VEGFR2 signalling. For example, TIMP2 can inhibit VEGF-A and FGF2 endothelial cell proliferation via a MMP independent mechanism involving increasing receptor tyrosine kinase association with an endogenous inhibitor SHP1, thereby preventing the downstream signalling that stimulates growth (Seo et al., 2003). TIMPs are often referred to as antiangiogenic but this may be a misconception as functional TIMPs are required to prevent tubule regression. For example, TIMP2 has been shown to suppress endothelial cell tube formation by targeting MT1-MMP but TIMP2 suppression destabilises vessels resulting in MMP1 dependent regression (Saunders et al., 2006). Similarly, Saunders et al. found that endothelial MT1-MMP, MT2-MMP and ADAM 15 dependent tube morphogenesis and invasion of collagen was inhibited by TIMP3, as was MMP1, MMP10, and ADAM15 dependent tube regression (Saunders et al., 2006). TIMP3 interacts with the extracellular matrix components such as heparan sulphate, but other TIMPs don't appear to have this ability therefore this may give TIMP3 a unique control mechanism that is important for its role in vessel stabilisation (Saunders et al., 2006). Hence, it is likely that TIMPs have a pluripotent role in angiogenesis.

1.6.11 A disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1)

A disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1) is generally recognised as an antiangiogenic factor (Iruela-Arispe et al., 2003). The

role of ADAMTS1 has been investigated in the normal female reproductive tract. In the female reproductive system, ADAMTS1 is essential for ovarian vascular medullary formation as ADAMTS1 null mice exhibit severely dysplastic or disorganised vascular networks (Shozu et al., 2005). ADAMTS1 null mice are also infertile in part due to their impared ovulation (Mittaz et al., 2004). ADAMTS1 expression has been found in the normal and decidual endometrium. Ng et al. who showed that ADAMTS1 expression was restricted to glandular epithelial cells and stromal cells surrounding the spiral arteries in the secretory endometrium (Ng et al., 2006). They provided evidence to suggest that ADAMTS1 is involved in cytokine regulation of endometrial decidualisation (Ng et al., 2006). Research by Wen et al. also showed that the expression of ADAMTS1 by endometrial stromal cells is under the control of gonadal steroids DHT and progesterone (Wen et al., 2006). ADAMTS1 has also been shown to be essential for healthy follicle growth which has been attributed to its metalloproteinase properties (Brown et al., 2006).

A disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1) (Kuno et al., 1997) is so called because it is closely related to proteins in the a disintegrin and metalloproteinase (ADAM) family and has a region which has 40% similarity to thrombospondin 1 and 2 containing three TSP type 1 motifs (Kuno et al., 1997). The TSP1 region in ADAMTS1 binds to heparin, as does this region in the TSP1 protein, indicating that it is functional (Kuno et al., 1997). In addition to the three TSP1 repeats and metalloprotease region, ADAMTS1 consists of a SP region, Prodomain, Disintegrin, Cysteine-Rich region and spacer region, as shown in Fig.12 (Iruela-Arispe et al., 2003).

Fig.12. Structure of ADAMTS1. ADAMTS1 contains SP region, Prodomain, Metalloprotease, Dinsintegrin, three Thromobospondin repeats (TSR), Cysteine-Rich region and a Spacer region. Adapted from Luque et al. (2003).

The 121kDa full length ADAMTS1 requires cleavage of the prodomain from the zymogen for activation (Rodriguez-Manzaneque et al., 2002). The active full length ADAMTS1 is then further cleaved after the second TSR and then the first TSR (Rodriguez-Manzaneque et al., 2002). This releases 86kDa and 65kDa fragments named N and C terminus fragments. Unlike the ADAM proteins, ADAMTS1 does not have a transmembrane binding domain but is instead a secreted protein (Iruela-Arispe et al., 2003).

ADAMTS1 was originally identified as a protein regulated by inflammatory mediators LPS and IL-1 in a murine colon adenocarcinoma cell line (Kuno et al., 1997). Extracellular matrix proteoglycans aggrecan and versican were found to be substrates for ADAMTS1 in its role as a metalloproteinase (Rodriguez-Manzaneque et al., 2002; Sandy et al., 2001). Recently ADAMTS1 was found to cleave transmembrane proteoglycan Syndecan 4 near the N terminus GAG site from the cell surface thereby regulating mouse lung endothelial cell adhesion and enhancing migration (Rodriguez-Manzaneque et al., 2009). In addition, the TSP1 region of ADAMTS1 was found to be involved in the cleavage and activation of antiangiogenic thrombospondin peptides (Lee et al., 2006) and the metalloprotease inhibitor Matrix Inhibitor Tissue Factor Pathway Inhibitor-2 can be cleaved from the cell membrane by ADAMTS1 (Torres-Collado et al., 2006).

The mechanisms by which ADAMTS1 interacts and regulates the vasculature have been investigated in vitro and in vivo. Recently, the invasion of endothelial cells into matrigel was shown to require ADAMTS1 expression (Su et al., 2008). ADAMTS1 has been shown to inhibit VEGF-A induced angiogenesis through different mechanisms (Iruela-Arispe et al., 2003). In one angiogenesis inhibitory mechanism ADAMTS1 can sequester VEGF-A with two TSP repeats and a spacer region and prevent its binding to the VEGFR2 receptor thereby inhibiting human aortic endothelial cell proliferation (Luque et al., 2003). Alternatively, ADAMTS1 inhibits angiogenesis by facilitating the cleavage of antiangiogenic thrombospondin peptides

which can inhibit FGF2-driven bovine aortic endothelial cell proliferation (Lee et al., 2006).

A detailed study in vivo by Lui et al. found that the antiangiogenic role of ADAMTS1 is dependent on its cleaved N and C terminal fragments (Liu et al., 2006). In a Lewis lung carcinoma mouse tumour model, ADAMTS1 N and C terminal fragments inhibited tumour proliferation, metastasis and angiogenesis (Liu et al., 2006). Studies in vitro suggested that this was via a mechanism inhibiting the shedding of HB-EGF and hence EGFR phosphorylation and ERK1/2 activation (Liu et al., 2006). In contrast, the full length active form of ADAMTS1 promoted tumour metastasis and had a weak positive affect on tumour proliferation and angiogenesis in vivo and promoted tumour cell invasion through matrigel in vitro (Liu et al., 2006). Of note, the full length form of ADAMTS1 was the predominant form in LLC conditioned medium perhaps due to the fact that in vivo, heparin inhibits ADAMTS1 cleavage (Liu et al., 2006). This demonstrates that the form of ADAMTS1 is critical for determining its function.

ADAMTS1 can be upregulated by growth factor treatment, such as VEGF-A, in endothelial cells (Xu et al., 2006). Apart from VEGF-A, factors regulating the expression and secretion of ADAMTS1 are relatively unknown however, HDAC6 has been found to prevent the expression of ADAMTS1 in human lung carcinoma cells (Chou and Chen, 2008). Protein kinase C was found to be involved in the upregulation of ADAMTS1 in endothelial cells (Xu et al., 2006). Recently, the role of early response gene ERG in the regulation of ADAMTS1 was discovered and associated with prostate cancer progression (Carver et al., 2009). This research suggests that ADAMTS1 gene expression could be controlled by ERG and HDAC in combination. This thesis will focus on angiogenic factors FGF2, CXCL1 and CXCL8 together with the antiangiogenic factor ADAMTS1.

1.7 Intracellular signalling molecules regulating endothelial cell function

1.7.1 Extracellular regulated kinase 1/2 (ERK1/2)

The networks of mitogen-activated protein kinases (MAPKs) are involved in many cellular processes such as cell survival, proliferation, migration and differentiation (Pearson et al., 2001). As shown in Fig. 13, MAPK cascades, involving phosphorylation of sequential proteins, all share similar components falling into the following categories, an initiation kinase (MAP3Ks/MEKK), a MAPKKinase (MAPKK/MEK) and a MAPKinase (MAPK). The activated terminal MAPKinases phosphorylate serine/threonine sites on target proteins (Pearson et al., 2001). These target proteins are often transcription factors such as c-fos, c-jun and SP-1(Turjanski et al., 2007). In this way, the MAPKinase cascades mediate many signal transduction events (Pearson et al., 2001).

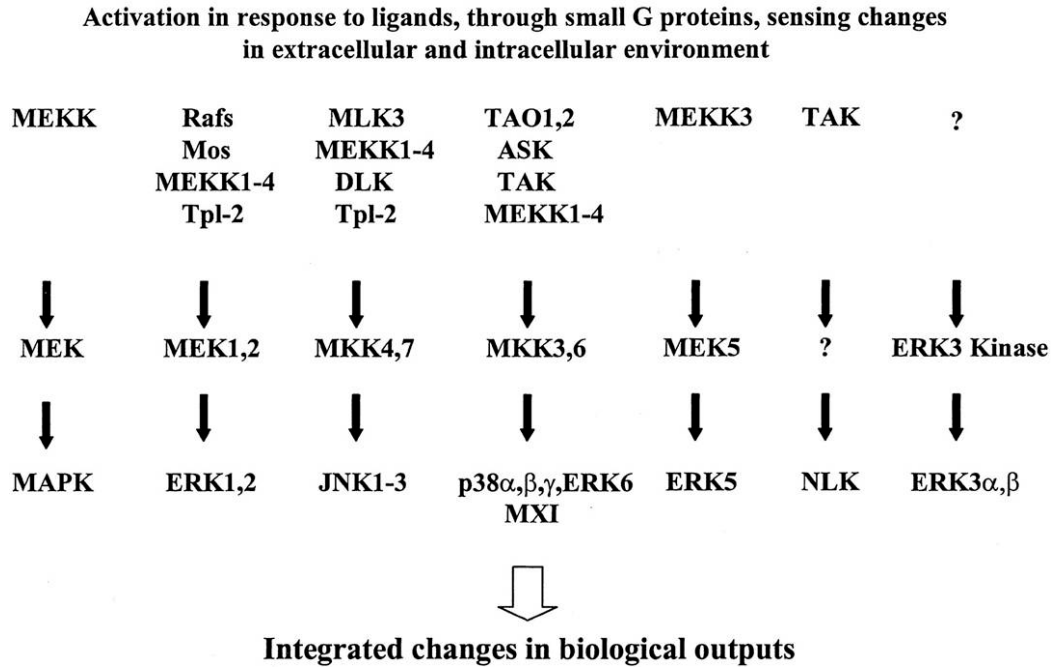


Fig. 13. The MAPKinase signalling cascades. Adapted from Pearson et al. (2001).

ERK1/2 is part of the classical mitogen cascade which consists of the initiating kinase Raf-A, Raf-B or Raf-C, the MAPKK MEK1/2 and subsequent activation of

the MAPK ERK1/2. Although ERK1/2 is a signalling kinase and not an angiogenic factor, it is involved in proangiogenic signalling of endothelial cells. ERK1/2 is activated by a phosphorylation cascade after angiogenic ligand-receptor binding (Hood et al., 2003). In the normal endometrium, ERK1/2 phosphorylation is highest in glandular epithelial cells of the late proliferative phase and secretory phase (Murk et al., 2008). In stromal cells ERK1/2 phosphorylation is also high in late proliferative phase but decreases slightly during secretory phases (Murk et al., 2008). This coincides with expression of inflammatory cytokines CXCL8 and COX-2.

The ERK1/2 MAPK cascade is initiated by an extracellular stimulus such as growth factor binding to its tyrosine kinase or G-protein coupled receptor (GPCR) (McKay and Morrison, 2007). Guanine nucleotide exchange factor activation by cellular receptors generates Ras GTP (Marshall, 1996). Ras proteins are anchored to the inner surface of the plasma membrane where Ras GTP subsequently activates the Raf family of MAP3kinases (Vögler et al., 2008). The Raf family consists of three alternatively spliced isoforms A, B and C. The Raf isoform that activates downstream effectors depends on the external stimulus and the cell type. For example in endothelial cells, C-Raf is activated by FGF2 signalling whereas in ventricular myocytes TPA activates primarily A-Raf (Wellbrock et al., 2004). Phosphorylation of MEK1/2 on two serine sites, S217 and S221, by Raf activates MEK1/2. MEK1/2 is the only known activator of ERK1 and ERK2 which are activated by phosphorylation of theronine and tyrosine sites within their activation segments (Wellbrock et al., 2004). The Ras-Raf-ERK1/2 signalling pathway is involved in endothelial cell function and angiogenesis (Depeille et al., 2007). For example, Hood et al. found that VEGF-A and FGF2-induced CAM angiogenesis was inhibited by ERK1/2 inhibitor PD98059 as well as infection of retroviral vector encoding mutationally inactive forms of Ras or C-Raf (Hood et al., 2003). Recently Rap1a, a Ras family small GTPase that can activate Raf, was found to be critical for microvascular endothelial cell FGF2-induced ERK1/2 activation, tube formation, migration, permeability and proliferation and matrigel plug angiogenesis (Yan et al., 2008). Similarly inhibition of ERK1/2 prevents 3D microvascular endothelial cell

tube formation, transcription factor Erg-1 activation and matrix metalloproteinase MT1-MMP and MMP2 expression suggesting that ERK1/2 mediates endothelial cell invasion of extracellular matrix (Boyd et al., 2005).

Activation of ERK1/2 can take place on scaffold proteins which help to compartmentalise MAPK signalling and facilitate the phosphorylation cascade (Brown and Sacks, 2009). For example, the scaffold protein kinase suppressor of Ras (KSR) present at the plasma membrane, binds to Raf, MEK1/2 and ERK1/2 and their proximity enables the rapid activation of ERK1/2 (Brown and Sacks, 2009). ERK1/2 dimers bound to KSR subsequently target cytosolic proteins as opposed to ERK monomers, not bound to KSR, which translocate to the nucleus (Brown and Sacks, 2009). Therefore, ERK1/2 activation can be regulated through the availability of these scaffold proteins (Brown and Sacks, 2009).

Regulation of ERK1/2 can also take place through dephosphorylation of the threonine and tyrosine activation sites by protein phosphatases (Raman et al., 2007). Phosphatases that act specifically on MAPKs are known as MAPK phosphatases (MKPs) (Raman et al., 2007). A specific ERK2 phosphatase is MKP3. Inactive MKP3 forms a complex with active ERK2 (Zhou et al., 2006). This enables ERK2 to activate MKP3 which subsequently dephosphorylates the Thr^{183} and Tyr^{185} of ERK2 (Zhou et al., 2006). Alternatively, ERK1/2 activity can be regulated by the non-specific protein phosphatase 2A (PP2A). PP2A can enhance ERK1/2 phosphorylation indirectly by dephosphorylating inhibitory sites on KSR and Raf (Raman et al., 2007). On the other hand, PP2A can directly dephosphorylate and inactivate ERK1/2 (Van Kanegan et al., 2005). In rat neuronal cultures, the calcineurin inhibitor cyclosporin A (CsA) inhibited ERK2 dephosphorylation after glutamate treatment suggesting that the phosphatase calcineurin (PP2B) may also regulate ERK2 phosphorylation by indirect or direct mechanisms (Paul et al., 2003).

Once activated by phosphorylation, ERK1/2 can phosphorylate cytoplasmic targets. For example, ERK1/2 can phosphorylate and inactivate tuberous sclerosis complex 2

(TSC2) (Lee and Hung, 2007). Active TSC2 binds to TSC1 and the TSC1/TSC2 complex is involved in inhibiting mTOR activation thereby preventing cellular proliferation (Guertin and Sabatini, 2007). ERK1/2 inhibition of TSC2 relieves the inhibitory effect of TSC1/2 on the mTOR pathway and allows cellular proliferation to proceed (Drakos et al., 2008).

Nuclear targets of ERK1/2 include transcription factors SP1, MSK, RSK and c-fos (Turjanski et al., 2007). For example ERK2 can phosphorylate the ternary complex factor family member Elk-1 (Turjanski et al., 2007). Activated Elk-1 can then bind to promoter regions of specific genes as seen with c-fos, part of the AP-1 complex, (Monje et al., 2005) or serum response elements (SRE) (Whitmarsh et al., 1995) to enhance gene transcription. ERK1/2 can enhance the expression of vascular endothelial growth factor (VEGF-A) by phosphorylating SP1 in fibroblasts (Milanini-Mongiat et al., 2002).

The importance of the ERK1/2 pathway in regulating cell functions such as proliferation is highlighted by the fact that oncogenic mutations have been found in the Ras, Raf and MEK1/2 stages of this MAPK cascade along with ERK1/2 hyperactivation in cancers (Roberts and Der, 2007; Wellbrock et al., 2004). In fact, Ras is the most commonly mutated oncogene in cancer (Roberts and Der, 2007). Due to this fact, clinical trials of inhibitors of Ras, Raf and MEK1/2 are in progress (Roberts and Der, 2007).

1.7.2 Phosphatidyl inositide 3 kinase

In addition to its activating Raf at the start of the ERK1/2 cascade, Ras can also directly activate phosphoinositide-3-kinase (PI3K) in vitro (Fig.14) (Marshall, 1996). Alternatively PI3K can be phosphorylated directly by binding to receptor tyrosine kinases through the SH2 domains (Fig.14) (Vivanco and Sawyers, 2002). There are three classes of PI3K of which class I_A is most commonly involved in tumourigenesis (Engelman, 2009). PI3K is made up of two subunits, p85α the regulatory subunit and p110 the catalytic subunit as shown in Fig.14.

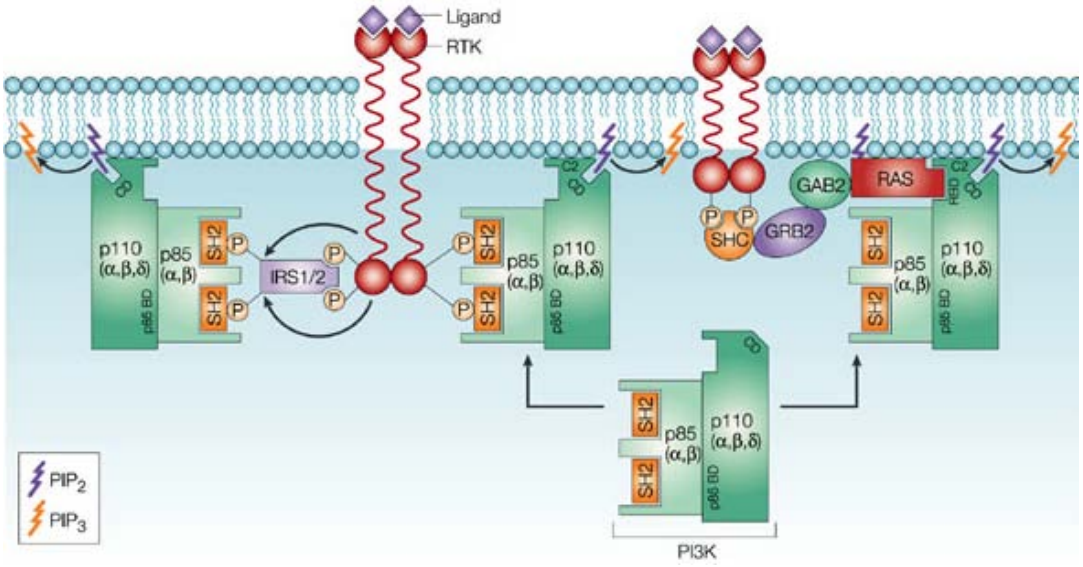


Fig.14. PI3K activation by growth factor receptors at the inner plasma membrane. Adapted from Engelman (2009). PI3K can be activated directly by receptor tyrosine kinases after ligand binding through phosphorylation of the SH2 regions of the p85α regulatory subunit. Alternatively phosphorylation of p85α can be mediated by an intermediate phosphoprotein such as insulin receptor substrates 1/2 (IRS1/2). Further indirect activation of PI3K can take place with the binding of Ras to the catalytic subunit p110.

Once the PI3K catalytic subunit p110 becomes active, its close proximity to the lipid substrates in the plasma membrane allows the generation of phosphatidylinositols PIP_3 from PIP_2 (Engelman, 2009). Interestingly, the PIP phosphatase PTEN, which converts PIP_3 back to PIP_2 is often mutated in cancer, including endometrial cancer, suggesting the importance of this PI3K pathway in tumourigenesis (Cully et al., 2006; Engelman, 2009; Kong et al., 1997; Vivanco and Sawyers, 2002). Of

particular interest is a recent research article which highlights the fact that in endometrial neoplastic cell lines insulin receptor substrate 2 (IRS2) expression is often upregulated when cells are haploinsufficient for PTEN (Szabolcs et al., 2009). In vivo studies by Szabolcs et al. suggest that IRS2 upregulation, in addition to PTEN loss of expression, enhances tumour growth either through the Akt/TSC/mTOR pathway (see section 1.7.3) or an alternative pathway (Szabolcs et al., 2009).

Once generated, PIP_3 recruits the cytoplasmic kinase AKT (protein kinase B) (Engelman, 2009). Subsequently, AKT is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 and -2 (PDK1 and PDK2) on Thr308 and Ser473 for maximal activation (Vivanco and Sawyers, 2002). AKT can be dephosphorylated by the previously mentioned phosphatase PP2A. The most studied roles of AKT involve its regulation of tumour cell survival, proliferation and growth (Vivanco and Sawyers, 2002). For example, AKT can directly phosphorylate the apoptotic factor BAD, preventing BAD's interaction and inhibition of proapoptotic factor Bcl-xl, thereby promoting cell survival (Vivanco and Sawyers, 2002). Additionally, AKT can phosphorylate the oncogenic protein MDM2 (Vivanco and Sawyers, 2002). MDM2 is responsible for promoting cell proliferation by targeting cell death factor p53 for ubiquitin degradation. AKT can also directly phosphorylate the central regulator of growth mammalian target of rapamycin (mTOR) on Ser^{2448} (Nave et al., 1999). Phosphorylation of mTOR is required for AKTs role in proliferation to be elicited (Engelman, 2009).

1.7.3 Mammalian target of rapamycin (mTOR)

As mentioned above, mTOR activation is an important terminus of the PI3K-AKT pathway. mTOR proteins forms two multiprotein complexes: mTORC1, with Raptor and PRAS40 (GβL), and mTORC2, with Rictor, mSIN1 and Protor proteins (Bjornsti and Houghton, 2004). However, the precise role of each of these proteins has yet to be defined. Raptor appears to have inhibitory actions on mTOR under nutrient

deficient conditions but may also act as a scaffold protein to enhance mTOR function (Bjornsti and Houghton, 2004). As shown in Fig. 15, absence of Raptor inhibition allows mTOR to phosphorylate substrates such as translation initiation factor 4E-BP1 thereby enhancing proliferative signals (Bjornsti and Houghton, 2004). mTOR can also phosphorylate p70S6K1 to enhance the translation of ribosomal proteins (Fig. 15) (Vivanco and Sawyers, 2002).

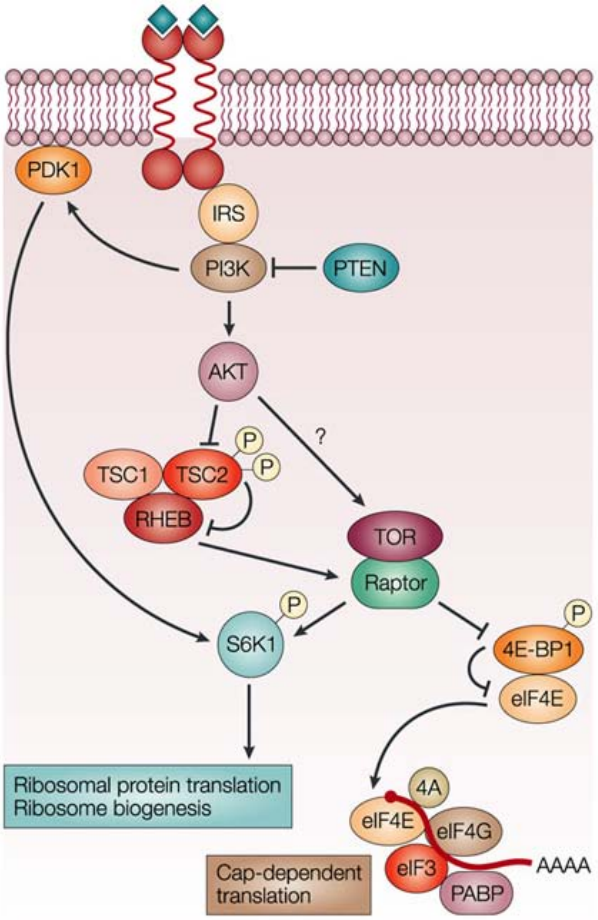


Fig. 15. The mTOR pathway activated by tyrosine kinase receptor ligand binding. Adapted from Vivanco et al. (2002). PI3K binding to IRS results in AKT activation leading to mammalian TOR (mTOR) activation and inhibition of mTOR inhibitor complex TSC1/2. TSC1/2 inactivation allows RHEB to relieve the inhibitory effect of Raptor on mTOR. mTOR subsequently regulates protein translation via phosphorylation of 4E-BP1and S6K1.

mTORC1 and mTORC2 have different regulatory roles. For example, in endothelial cells, mTORC2 may regulate AKT as rapamycin, the inhibitor of mTOR, can inhibit AKT phosphorylation in vivo (Guertin and Sabatini, 2007). In contrast, mTORC1 is thought to be downstream of AKT as AKT can phosphorylate TSC1/2 and PRAS40

(Guertin and Sabatini, 2007). TSC1/2 can inhibit mTOR activity through the regulation of small GTP protein RHEB, see Fig. 15. RHEB is required for mTOR activity and this may be because it induces a conformational change in mTOR (Wullschleger et al., 2006).

mTOR is also activated by additional pathways such as the ERK1/2 MAPKinase pathway. As previously mentioned ERK1/2 can phosphorylate TSC2 and relieve its inhibitory effect on mTOR (Drakos et al., 2008). In HEK293T cells, evidence suggests that the mTOR pathway may additionally be regulated by the binding protein 14-3-3β, because 14-3-3β can bind via serine 1210 to phosphorylated TSC2 resulting in an increase in S6K1 phosphorylation (Shumway et al., 2003).

In vivo studies demonstrate that the mTOR inhibitor, rapamycin, can inhibit angiogenesis and enhance blood vessel permeability induced by VEGF-A (Phung et al., 2006). In addition rapamycin reduces xenograft tumour growth and vascular permeability suggesting that it could be a useful inhibitor for cancer therapy (Phung et al., 2006). A recent phase 3 trial to investigate the effectiveness of mTOR inhibitor Temsirolimus, in patients with mantel cell lymphoma, indicate that inhibition of mTOR may be an effective method to increase patient survival rates (Hess et al., 2009).

1.7.4 Cellular Src (c-Src)

Src was first identified as a viral protein v-Src and later found to be an endogenous cellular protein, c-Src (Parsons and Parsons, 2004). c-Src (Src) is a ubiquitously expressed member of the Src-family nonreceptor tyrosine kinases (SFKs) which also include Fyn and Yes (Parsons and Parsons, 2004). SFKs are attached through their N terminus to the cytoplasmic membrane from where they interact, through their Src homology (SH)2 domain, with tyrosines of activated receptor tyrosine kinases (Bromann et al., 2004) and GPCRs. The activation of Src during recruitment to receptor tyrosine kinases such as EGFR may involve small GTPases Ras and

Ral.(Bromann et al., 2004). Src can be activated by tyrosine kinase fibroblast growth factor receptor 1 (FGFR1) in endothelial cells (Klint et al., 1999).

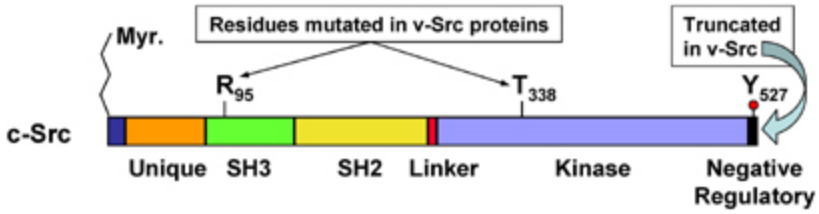


Fig. 16. Src domain structure. Adapted from Parsons et al. (2004).

After tyrosine kinase activation Src can activate downstream signalling pathways leading to ERK1/2, PI3K, PKC and STAT3 activation (Bromann et al., 2004; Deo et al., 2002).

GPCRs for ligands such as LPA, angiotensin and endothelin cause the activation of Src (Luttrell and Luttrell, 2004). Src can interact directly with GPCRs by binding to the GPCRs intracellular domains or indirectly by binding to their associated G proteins and to scaffold proteins such as β-arrestin (Luttrell and Luttrell, 2004). Research by Cao et al. found that the β3 adrenergic receptor can directly bind Src and this binding is essential for stimulation of ERK1/2 (Cao et al., 2000). Src is also involved in the transactivation of tyrosine kinase receptors by GPCRs (Petreaca et al., 2007). For example, Petreaca et al. found in microvascular endothelial cells that inhibition of Src with the VEGFR2 inhibitor SU6656 also abolished CXCR2/VEGFR2 complex formation and VEGFR2 phosphorylation, after treatment with CXCR2 ligand CXCL8 (Petreaca et al., 2007). Also, after prostaglandin $F_{2\alpha}$ stimulation of FP receptor GPCR, Src may be involved in the transactivation of the tyrosine kinase EGFR leading to ERK1/2 stimulation (Jabbour et al., 2006b). Stimulation of endothelial cells with soluble IL6 receptor utilises Src in a mechanism upregulating cytokine CXCL1 (Matsumiya et al., 2002).

Src has an essential role in growth factor stimulated endothelial cell function and angiogenesis. For example, Src activation was found to be essential for VEGF-induced in vivo matrigel plug angiogenesis and vascular permeability (Eliceiri et al.,

1999). A recent article by Gavard et al. demonstrated that VEGFR2 activation and regulation of endothelial cell junctions requires Src for β-arrestin mediated VE-cadherin internalisation (Gavard and Gutkind, 2006). Additionally, VEGF-A/integrin αvβ5 mediated chick chorioallantoic membrane (CAM) angiogenesis Src was required for Raf-ERK1/2 activation (Hood et al., 2003). In immortalised brain capillary endothelial cells, FGF-2 treatment can stimulate Src activation as part of an essential step in endothelial network formation and proliferation (Klint et al., 1999; Larsson et al., 1999). Src, along with ERK1/2, is also essential for vascular lumen formation in vitro (Koh et al., 2009). Similarly, Kilarski et al. found that Src is required for FGF2-induced CAM angiogenesis in vivo (Kilarski et al., 2003). Furthermore, Lui et al. found, using time-lapse microscopy, that microvascular endothelial cord formation in collagen matrix was inhibited by the Src inhibitor PP2 (Liu and Senger, 2004).

1.7.5 Nuclear factor of activated T cells (NFAT)

NFAT family members are activated when dephosphorylated by calcineurin (Rao et al., 1997). This allows NFAT to translocate to the nucleus where it regulates gene transcription (Rao et al., 1997). In endothelial cells, NFAT has been found to be involved in the transcription of growth factors and cytokines. For example, histamine treatment of HUVECs induced the NFAT-dependent expression of CXCL8 (Boss et al., 1998). Cyclosporin A (CsA) is a commonly used inhibitor of calcineurin and therefore used to examine the effects of preventing NFAT activity. In vitro, CsA can inhibit VEGF-A induced nuclear translocation of the transcription factor NFATc1 in human intestinal microvascular endothelial cells (HIMECs) and humon pulmonary valve endothelial cells (HPVECs) (Johnson et al., 2003; Rafiee et al., 2004). Similarly, CsA inhibits VEGF-A-induced endothelial cell migration, proliferation and network formation (Hernandez et al., 2001; Johnson et al., 2003; Rafiee et al., 2004). In an in vivo model of angiogenesis, the rat corneal angiogenesis assay, CsA treatment inhibited VEGF-A-induced, but not FGF2-induced, vessel formation (Hernandez et al., 2001). NFAT transcription may regulate COX-2 expression and prostaglandin biosynthesis in endothelial cells as

CsA inhibits VEGF-A-induced COX-2 expression and NFAT binding to the COX2 promoter (Hernandez et al., 2001). In addition CsA inhibition of VEGF-A induced angiogenesis could be significantly restored by PGE_2 treatment (Hernandez et al., 2001).

1.7.6 Regulator of calcineurin (RCAN)

RCAN1 is also known as Down syndrome critical region 1 (DSCR1), Adapt78 and MCIP1 (Davies et al., 2007). RCAN1 is located on chromosome 21 and individuals with Down's syndrome have an extra copy of this chromosome (Fuentes et al., 2000). Individuals with Down's syndrome have lower rates of many cancers (Ryeom et al, 2008). A recent paper by Baek et al. has provided evidence to show that the overexpression of RCAN1 in Down's syndrome can suppress tumour growth (Baek et al., 2009). RCAN 1 is an inhibitor of calcineurin. By binding to calcineurin and preventing the calcineurin-dependent dephosphorylation of NFAT, RCAN1 can impede NFAT induced gene transcription. Recently, the RCAN1-4 isoform was identified as a negative regulator of NFAT dependent CXCL8 expression (Maldonado-Perez et al., 2009).

RCAN1 has 4 splice variants from exons 1, 2, 3 and 4 (Harris et al., 2005). Although exon 2 is thought to be non functional and exon 3 only contains 3 aminoacids, RCAN1-exon1 (RCAN1-1) and RCAN1-exon4 (RCAN1-4) are expressed by endothelial cells and affect endothelial cell function (Qin et al., 2006). For example, a study by Minami et al. showed that RCAN1-4 can inhibit NFAT induced VEGF-A expression in HUVECs (Minami et al., 2004). The overexpression of RCAN1-4 inhibits VEGF-A induced HUVEC network formation and proliferation (Minami et al., 2004). Further in vivo studies using a matrigel plug assay and melanoma xenografts indicate that VEGF-A induced angiogenesis is inhibited by RCAN1-4 overexpression (Minami et al., 2004). This suggests that RCAN1-4 has anti-angiogenic actions. In contrast RCAN1-1 (aka DSCR1-1L) appears to have pro-angiogenic properties (Qin et al., 2006). A study by Qin et al. showed that RCAN1-1 siRNA inhibited VEGF-A induced HUVEC proliferation and in vivo matrigel plug

angiogenesis (Qin et al., 2006). This inhibition in RCAN1-1 was suggested to be due to a decrease in NFAT activity since NFAT activity was reduced with RCAN1-1 siRNA and cyclosporin A inhibited RCAN1-1 induced angiogenesis (Qin et al., 2006). Alternatively, the RCAN1-Like1 (RCAN1-L1) isoform has been shown to inhibit NFAT activation as well as unstimulated HUVEC network formation and VEGF-A induced HUVEC proliferation (Gollogly et al., 2007).

1.8 Aims and objectives of the thesis

In this chapter, the role of prostaglandins in endometrial adenocarcinoma has been reviewed along with the role of growth factors in angiogenesis and endothelial cell function. FP receptor expression is upregulated, along with angiogenic factor expression, in endometrial adenocarcinoma. Also an increase in microvascular density in endometrial cancers is associated with a poorer prognosis for patients. Research into the factors mediating the initiation and progression of endometrial adenocarcinoma, such as those regulating angiogenesis, could lead to the identification of appropriate therapeutic targets. Further studies are needed, in the context of endometrial adenocarcinoma, in order to elucidate the mechanisms by which the autocrine and paracrine actions of factors, produced by prostaglandin $F_{2\alpha}$-FP receptor signalling, regulate endothelial cell function and angiogenesis. Therefore, the specific aims of the research presented herein were to:

1. Determine the expression and localisation of antiangiogenic ADAMTS1 in endometrial adenocarcinoma and investigate its regulation by prostaglandin $F_{2\alpha}$ signalling. This was performed using normal and cancer endometrial tissue samples and the Ishikawa endometrial adenocarcinoma cell line, stably expressing the FP receptor to levels seen in cancer (FPS cells) as described in chapter 3.

2. Investigate the paracrine effects of proangiogenic factors FGF2, CXCL1 and CXCL8, secreted into conditioned medium from $PGF_{2\alpha}$ treated FPS cells (P CM), on endothelial cell functions of differentiation and proliferation. This was performed using conditioned medium and human umbilical vein endothelial cells (HUVECs) in network (differentiation) formation and proliferation assays as described in chapter 4.

3. Investigate the role of prostaglandin $F_{2\alpha}$ and the FP receptor in the regulation of endothelial cell network formation and proliferation. This was achieved by assessing endothelial cell network formation and proliferation after treatment with exogenous $PGF_{2\alpha}$ or P CM, in combination with the FP receptor inhibitor AL8810, and silencing of the FP receptor using short hairpin RNA, as described in chapter 5.

4. Identify the role of antiangiogenic ADAMTS1 and regulator of calcineurin (RCAN1-4) in P CM-induced endothelial cell network formation and proliferation. This was conducted using viral vector gene silencing and overexpression of RCAN1-4 and small interference RNA silencing of ADAMTS1 in order to investigate the effects on P CM-induced endothelial cell network formation and proliferation as described in chapter 6.

5. Investigate the role of prostaglandin $F_{2\alpha}$-FP receptor signalling in angiogenesis in vivo. This was carried out by analysing microvessel density and angiogenic factor expression in the sponge/matrigel mouse model and tumour xenograft mouse model as described in chapter 7.

2 General materials and methods

2.1 Tissue collection

Endometrial cancer tissue and normal tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/1999/6/4. Written informed consent was obtained from all subjects prior to tissue collection. Endometrial cancer tissue was obtained from women undergoing surgery for removal of endometrial cancer and who had been pre-diagnosed on endometrial biopsy to have endometrial adenocarcinoma of the uterus of the endometrioid type. The median age of patients was 60.5 years. Cancer biopsies were assessed by a pathologist and assigned a grade, well differentiated (grade 1; n=10), moderately differentiated (grade 2; n=10) or poorly differentiated (grade 3; n=10). Normal endometrium from the proliferative phase and early-mid-late secretory phases of the menstrual cycle (n=10), was collected with an endometrial suction curette from women undergoing surgery for gynecological procedures including surgical sterilisation or abnormal uterine bleeding in whom histological examination of endometrium was normal with no underlying endometrial pathology (Pipelle, Laboratoire CCD, France). The median age of women was 30.5 years. Biopsies were dated according to stated last menstrual period (LMP) and confirmed by histological assessment (Sales et al., 2005). After collection, tissue was placed in RNAlater (Ambion) and stored at -70^{o}C (for RNA extraction) or fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis).

2.2 Cell Culture

2.2.1 Endothelial cells

Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, USA) were cultured in Endothelial Basal Medium (EBM-2) with 2% FBS and growth supplements (VEGF, FGF, PGDF, IGF, EGF, ascorbic acid, heparin and gentamycin) subsequently referred to as Endothelial Growth Medium (EGM) (Lonza, Walkersville, USA) at 37^{o}C in a 5% CO_2 atmosphere. Under experimental

conditions cells were incubated with EBM-2 plus 1% FBS with the addition of ascorbic acid and gentamycin aliquots (Lonza, USA). When cells reached 70-80% confluency, they were passaged by washing three times with Hank's balanced salt solution (HBSS), followed by a 5 minute incubation with trypsin. Trypsinisation was stopped by the addition of Trypsin Neutralising Solution (TNS) (1volume trypsin: 2volumes TNS) and cells were centrifuged in a 15ml tube at 200rpm for 5mins. Cells were counted with a haemocytometer and seeded at the required density prior to the start of an experiment. HUVEC of passages 3-8 were used for all experiments.

2.2.2 Ishikawa cells

A cell line stably overexpressing the FP receptor to levels seen in endometrial cancer was constructed by Cytomix Ltd. (Cambridge, United Kingdom) as described by Sales et al. 2005. Briefly, FP receptor cDNA was transfected into wild type Ishikawa cells (WT) at passage 10 in the sense direction. Individual cell populations were selected for with addition of 800µg/mL G418. The clone with the highest level of FP receptor expression, FP sense 32 (FPS), as determined by Western blot analysis and relative quantification of FP receptor expression, were expanded and stored in liquid nitrogen. Subsequently, WT and FPS Ishikawa cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley, UK) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin solution, at 37^{o}C in a 5% CO_2 atmosphere. Cells were passaged at a confluency of 80-90% by washing three times with PBS to remove medium and incubated with trypsin for 5mins at 37°C. DMEM was added to quench the trypsinisation, and cells were counted with a haemocytometer for use in subsequent experiments. Under experimental conditions cells were serum starved for 24hrs in DMEM containing only 1% penicillin/streptomycin and no FBS prior to treatment.

2.2.3 Reagents and cellular signalling inhibitors

Recombinant CXCL8 (CXCL8), recombinant CXCL1 (CXCL1), Arachidonic acid (AA), PGE_2, $PGF_{2\alpha}$, AH6809 and indomethacin were purchased from Sigma Chemical Co. (Dorset, UK). PD98059, SU4984, NS398, SB225002, LY294002, PP2, wortmannin and rapamycin were purchased from Calbiochem (Nottingham, UK). AL8810 and SC560 were purchased from Caymen Chemical Company (Michigan, USA). Recombinant FGF2 peptide was purchased from PeproTechEC Ltd. (London, UK).

Table 1. Concentration of inhibitors

Inhibitor	Molecules inhibited	Working concentration
AL8810	FP receptor	50μM
AH6809	EP1/EP2 receptor	50μM
YM254890	Gq protein	1μM
U73122	Phospholipase C-β	10μM
BAPTA-AM	Calcium chelator	50μM
Cyclosporin A	Calcineurin	1μM
W-7	Calmodulin	25μM
INCA6	NFAT	20nM
PP2	cSrc	10μM
AG1478	EGFR	200nM
PD98059	MEK	50μM
NS-398	COX-2	10μM
SC-560	COX-1	10μM
Indomethacin	COX	3μg/ml
SU4984	FGFR1	20μM
SB225002	CXCR2	350nM
Rapamycin	mTOR	100ng/ml
Wortmannin	PI3K	200μM
LY294002	PI3K	50μM

All inhibitors indicated in Table 1 were used at concentrations at or below their IC50 or working concentrations reported for other systems. The effect of inhibitors on endothelial cell viability was assessed with the proliferation assay (see section 2.9.2) using endothelial basal medium (EBM1%) plus inhibitor alone.

To elucidae the intracellular signalling pathways using chemical inhibitors, it is important to understand the mode by which the inhibitors prevent their target molecules downstream signalling. For example, AL8810 the antagonist of the FP receptor competes with $PGF_{2\alpha}$ for binding to the FP (Griffin et al., 1999). U73122 inhibits phospholipases, including PLC which is activated by agonist binding to the FP receptor, however the precise mechanism of the action of U73122 is unknown (Yule and Williams, 1992). AH6809, is a competitive antagonist of the prostaglandin E_2 EP1 and EP2 receptors (Coleman et al., 1994).

The membrane permeable calcium chelator BAPTA-AM sequesters calcium rendering it unable to initiate downstream signalling events (Tsien, 1981).
N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) is a calmodulin (CaM) antagonist that binds to multiple sites on Ca^{2+}-bound CaM via Van der Waals interactions and electrostatic interactions (Osawa et al., 1999). The binding of W-7 in the hydrophobic pocket of CaM, and the conformational change caused by W-7 binding, inhibits the interaction of CaM with target proteins, including calcineurin (Osawa et al., 1999). Cyclosporin A (CsA) is a commonly used inhibitor of calcineurin that indiscriminately blocks the activation of all calcineurin targets. CsA enters a cell and complexes with proteins called cyclophilins, enhancing cylophilins affinity for calcineurin (Kapturczak et al., 2004). The CsA-cyclophilin complex binds to calcineurin and prevents its activation of target proteins such as NFAT (Kapturczak et al., 2004). The inhibitor of NFAT-calcineurin association 6 (INCA6) is a selective inhibitor of the calcineurin-NFAT interaction. INCA6 binds to calcineurin Aα at cysteine 266 which is not in the core calcineurin-NFAT binding site (PXIXIT) (Kang et al., 2005). Therefore, the inhibition of NFAT activation by

INCA6 is regulated through an allosteric conformational change of the calcineurin PXIXIT docking site (Kang et al., 2005).

PP2(4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) preferentially interacts with Src-family protein tyrosine kinases (SFK) however, it can bind to other tyrosine kinases including C-terminal Src kinase (CSK) and SAPK2a/p38 (Chong et al., 2005). PP2 inhibits SFK activation by binding to the adenine pocket of the ATP-binding site and via its core hetero-bicyclic region thereby preventing SFK activation (Chong et al., 2005). The aryl substituent of PP2 inserts into the hydrophobic pocket adjacent to the adenine pocket (Chong et al., 2005).

AG1478 is a quinazoline synthetic inhibitor highly specific to EGFR. AG1478 competes with ATP to bind with EGFR, thereby preventing its activation, but does not prevent substrate binding (Levitzki and Gazit, 1995).

PD98059 binds to MEK1/2 (the activator of ERK1/2), at a site unrelated to substrate binding, causing a change in tertiary structure which prevents ATP binding and therefore MEK1/2 activation. Consequently, by inhibiting MEK1/2 activation, PD98059 prevents ERK1/2 activation (Alessi et al., 1995).

Indomethacin belongs to the structural class of indole and indene acetic acids and inhibits both COX-1 and COX-2 with similar efficacy (Mitchell and Warner, 2006). NS398 is a selective diarylheterocycle inhibitor for COX-2, as it has only a weak effect on COX-1 activity, and is therefore used as a COX-2 specific inhibitor (Walker et al., 2001). The binding of NS3983 to the active site of COX-2 results in a non-reversible conformational change that prevents substrate binding (Walker et al., 2001).

The specific COX-1 inhibitor, SC-560, belongs to the diarylheterocycles but lacks the phenylsulphonamide moiety common to the COX-2-selective inhibitors,

therefore SC-560 binding to the active site of COX-1 is selective but reversible (Walker et al., 2001).

Wortmannin is a selective inhibitor of PI3K that binds to the p110 catalytic subunit of PI3K and prevents the formation of PIP_3 from PIP_2 (Arcaro and Wymann, 1993). LY294002 is a selective inhibitor of PI3K that competes, with ATP, for the ATP binding site of PI3K (Vlahos et al., 1994). Rapamycin is an inhibitor of the mTOR complex 1 (mTORC1) consisting of mTOR, Raptor and mLST8. Rapamycin simultaneously binds to hydrophobic pockets of FKBP12 and the FRB domain of mTOR which may interfere with catalytic activity of mTOR by preventing phosphatidic acid binding (Ballou and Lin, 2008). Rapamycin may also interfere with the stabilisation of the mTOR-raptor complex and Rheb–GTP activation of mTOR (Ballou and Lin, 2008).

SU4984 is an inhibitor of the FGF2 receptor FGFR1 (Mohammadi et al., 1997). The oxindole portion of SU4984 occupies the FGFR1 site in which the adenine of ATP binds, whereas the moieties that extend from the oxindole contact residues in the hinge region of FGFR1 between its two kinase lobes (Mohammadi et al., 1997). SB225002 is an antagonist of the CXCR2 receptor which competes with CXCL1 and CXCL8 for binding (Catusse et al., 2003). The binding of SB225002 occurs through epitopes which are disseminated throughout CXCR2. The epitopes that SB225002 binds to differ according to the use of CXCL1 or CXCL8 as a competitor and do not necessarily overlap with the CXCL1 or CXCL8 binding sites (Catusse et al., 2003).

2.2.4 Conditioned medium

WT or FPS cells were seeded at a density of $2x10^6$ cells and incubated at 37^oC in a 5% CO_2 atmosphere to allow adherence before serum-starvation for 24hrs. Thereafter, cells were treated with 20mls of DMEM containing 8.4μM indomethacin in the presence of 100nM $PGF_{2\alpha}$ or vehicle for 24hrs to create $PGF_{2\alpha}$ conditioned medium (P) or vehicle conditioned medium (V). Conditioned medium from three

independent experiments was pooled, aliquoted and stored at -20°C until required. Fibroblast growth factor 2, CXCL1 and CXCL8 were immunoneutralised from the FPS cell $PGF_{2\alpha}$ conditioned medium (FP P CM) by overnight incubation with 0.5µg/ml FGF2 antibody, 2µg/ml CXCL1 antibody or 2µg/ml CXCL8 antibody. The FGF2 antibody recognising the LMW 18kDa isoform and the CXCL1 antibody were purchased from Santa Cruz Biotechnology (Autogen-Bioclear, Wiltshire, UK). The CXCL8 antibody was purchased from Sigma, UK. The immune complex was removed by 4hr incubation with 20-40µl of a 50% protein A plus G slurry (Calbiochem, Darmstadt, Germany). Conditioned medium immunoneutralised with Goat IgG was used as a control. Immunoneutralised CM was stored at -20°C until use. The FGF2, CXCL1 or CXCL8 protein content in the CM before and after immunoneutralisation was investigated by ELISA (see section 2.8.2 for further details). An advantage to using conditioned medium, as opposed to recombinant ligand alone, is that the concentration of proteins relative to each other, in the CM, is representative of the tumour environment (van Beijnum and Griffioen, 2005).

2.3 RNA analysis

2.3.1 RNA extraction from tissues and cells

2.3.1.1 Cells

RNA was extracted from cells using TRI-Reagent (Sigma-Aldrich, Dorset, UK), containing guanidine thiocyanate and phenol in a monophase solution, following maufacturers protocols. Briefly, the RNA, DNA and protein is dissolved in the solution following homogenisation or lysis. Cells were lysed by pipetting the TRI-Reagent up and down on the cell covered surface of culture dish. The TRI-Reagent-cell mixture was combined with 1-Bromo-3-choloropropane (BCP) and added to phase-lock tubes (Invitrogen) to allow for easy separation of the aqueous RNA phase from the DNA and protein. After 30seconds of shaking the phase-lock tubes to mix the solutions, the mixture was allowed to stand for 10mins at room temperature, to ensure complete dissociation of the nucleoprotein complexes, and then centrifuged for 15mins at 14000xg and 4°C. The colourless, upper aqueous phase (containing

RNA), was transferred to a fresh tube containing isopropanol and allowed to stand at room temp for 5-10mins followed by centrifugation at 14000xg for 15 mins at 4°C. The RNA formed a pellet at the bottom of the tube which was washed with 75% ethanol, dried and reconstituted in RNAse-free water by repeated pipetting.

2.3.1.2 Tissue

To purify Total RNA from tissue, the RNeasy Mini Kit (Qiagen,) was used according to manufacturer's instructions. For example, 30mg mouse kidney tissue was weighed and RNA was extracted for use as a positive control during Quantitative-PCR TaqMan reactions (see section 2.4). Tissue was homogenised in Buffer RLT (Qiagen) using a mechanical tissue homogeniser. The lysed tissue was centrifuged and the supernatant collected. This supernatant was transferred to a new microcentrifuge tube and combined with and equal volume of 70% ethanol. Up to 700µl of the ethanol mixture was added to an RNeasy spin column in a 2ml tube and centrifuged for 15seconds at 8000xg. This step was repeated until all the ethanol mixture had flowed through the spin column and the flow-through was discarded. The spin column membrane was washed with 700µl Buffer RW1, followed by two washes with 500µl Buffer RPE. To elute the RNA from the membrane, the spin column was placed in a new collection tube, 50µl of RNase free water was added and the column was centrifuged to release the reconstituted RNA.

2.3.2 RNA quantification

The concentration and purity of the reconstituted RNA was measured using the Nanodrop-1000 (Labtech International, East Sussex, UK) spectrophotometer. The concentration of RNA was expressed as ng/µl. RNA absorbance is measured at 260nm and proteins have a peak absorbance at 280nm. A 260/280 ratio of >1.7 indicated the RNA was free from protein and DNA contamination as protein contamination will reduce this ratio (Gallagher, 2001). Purified RNA was stored at -80°C.

2.4 TaqMan® quantitative RT-PCR (QPCR)

2.4.1 Preparation of cDNA from RNA by reverse transcription.

Firstly, cDNA reverse transcription was carried out on purified total RNA. RNA was diluted to a concentration of 100ng/µl in RNase-free water. A 20µl reaction mixture was made as using a mix of TaqMan Reverse Transcription Reagents (AppliedBiosystems); 2µl reverse transcription buffer, 5.5mM $MgCl_2$, 2mM dNTPs, 0.4U/ml RNase Inhibitor, 2.5 µM Random Hexamers, 2mM Oligo dT, 1.25U/ml Multiscribe Reverse Transcriptase, volume corrected to 16µl with H_2O. For each sample 4µl of 100ng/µl RNA was added to 16µl of the mix to create a 20µl total reaction volume. If a 100µl reaction volume was needed, quantities were increased by a factor of 5.

Reverse transcription was carried out using a Techne TC-512 Thermal Cycler (Techne Inc., Burlington, NJ, USA). The thermal profile parameters consisted of 1 cycle involving 1hour at 42°C, 5minutes at 99°C and 5minutes at 5°C with a 4°C hold step. The cDNA was stored at -20°C.

2.4.2 TaqMan® reaction

After reverse transcription, the cDNA samples were used to examine the levels of a particular gene of interest using a standard TaqMan reaction and the ABI7900HT Real-Time PCR System (AppliedBiosystems,FosterCity,CA USA). The level of ribosomal 18S was measured in each sample, as an endogenous control, using an 18S standard mix (TaqMan Ribosomal RNA Control reagents, VIC probe, AppliedBiosystems). The 18S mix was made using; 18S forward primers, 18S reverse primer and 18S probe (~ 840ul total) plus 1260 µl TE Buffer, mixed, aliquoted stored at -20°C. Primers and probes for target sequences were used at a concentration of 25nM and 10nM, respectively. The TaqMan reaction mixture was made using TaqMan Universal PCR Master Mix (2✕) (AppliedBiosystems). A 20µl TaqMan reaction mixture in duplicate was made using; 1X TaqMan Universal PCR Master Mix, forward primer and reverse primers (25nM), Probe (10nM), 18S

standard mix (50nM) and 2µl (40µg) cDNA. Additionally, negative control samples containing no reverse transcriptase enzyme or no cDNA were used. Samples were mixed and centrifuged before transferring to the wells of the TaqMan® Microamp Fast Optical 96 well reaction plate (AppliedBiosystems), in duplicate 23µl reaction volumes. The TaqMan reaction volume was set as 20µl on the ABI7900 machine.

2.4.3 Principles of the TaqMan® quantitative RT-PCR (QPCR) reaction

TaqMan uses a fluorogenic probe to allow detection of a polymerase chain reaction (PCR) product as seen in Fig. 17. The forward and reverse primers amplify a specific sequence of cDNA in a polymerase chain reaction (PCR) and the fluorogenic probe hybridises with the PCR product to generate a fluorescent signal (Fig. 17). An internal control of ribosomal 18S standard is added to the reaction mixture to normalise for the variation in reaction mixture volume and total RNA concentration (see section 2.3.2). The formation of the PCR product is measured, in real time, at multiple time points during the reaction hence Real Time PCR (RT-PCR) (see Relative Quantitation Using Comparative CT Getting Started Guide, AppliedBiosystems 7900HT Real-Time PCR System, www3.appliedbiosystems.com for further details.)

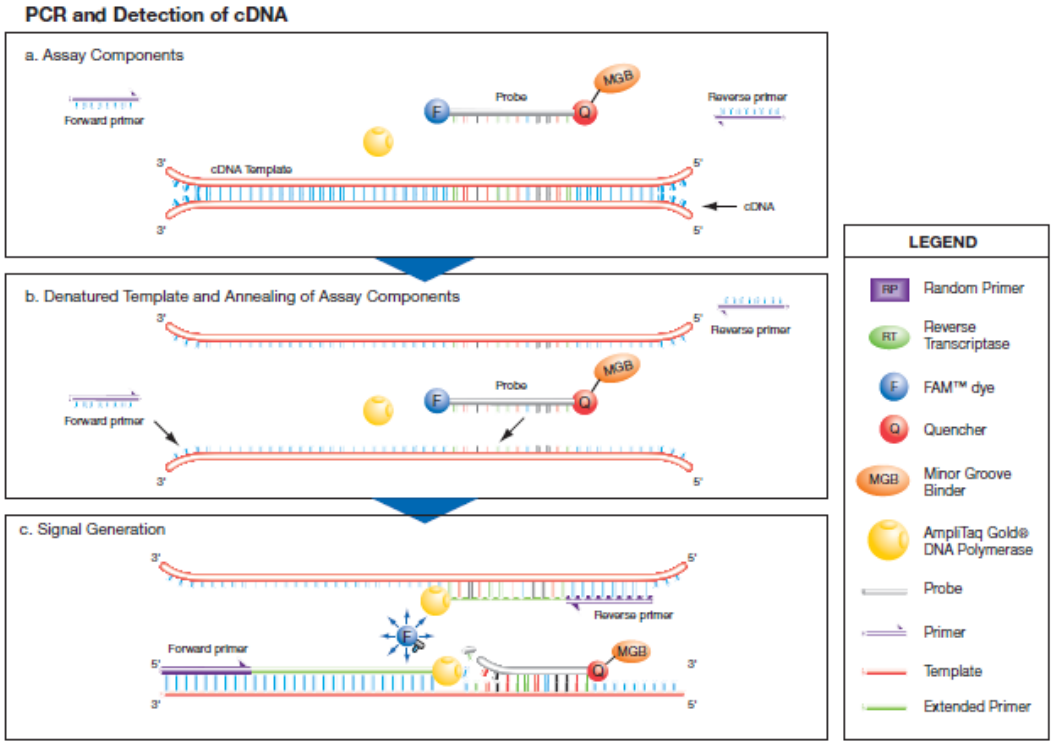


Fig. 17. A schematic representation of the TaqMan reaction. The Two-Step RT-PCR method was used in which total RNA was reverse transcribed (RT) into cDNA followed by Real Time PCR (adapted from).

2.4.4 Analysis of results

Comparative analysis of mRNA quantities was carried out by determining the relative quantitation (RQ). RQ is the quantity of a single nucleic acid target sequence within an unknown sample relative to the quantity in a calibrator sample. In this thesis, the calibrator sample was a normal endometrial sample when looking at gene expression in normal endometrium and cancer. In cell culture studies, the calibrator sample was either an untreated control or a sample at time zero in a time-course study. In Real-Time PCR the levels of the target sequence are measured at multiple time points throughout the reaction creating an amplification curve, as shown in Fig. 18.

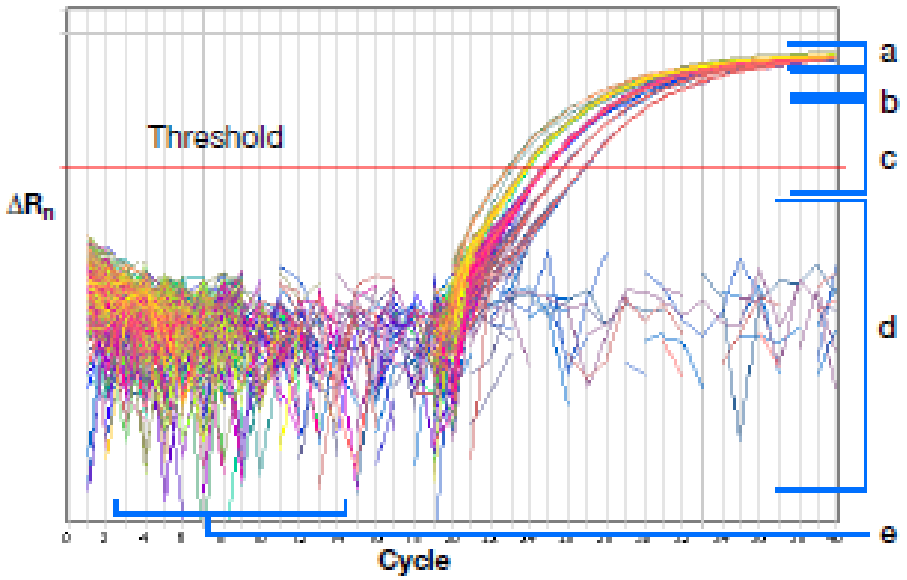


Fig. 18. Amplification curve data from a TaqMan assay. (adapted from Relative Quantitation Using Comparative CT Getting Started Guide, AppliedBiosystems 7900HT Real-Time PCR System, www3.appliedbiosystems.com).

As shown in Fig. 18, a typical amplification curve has a: Plateau phase (a), Linear phase (b), Exponential phase (c) - The exponential growth phase of the amplification curve, Background (d) – Any reading that falls below the threshold and is not part of an amplification curve and Baseline (e) – The initial cycles of the reaction before the target sequence is detected, by fluorescence.

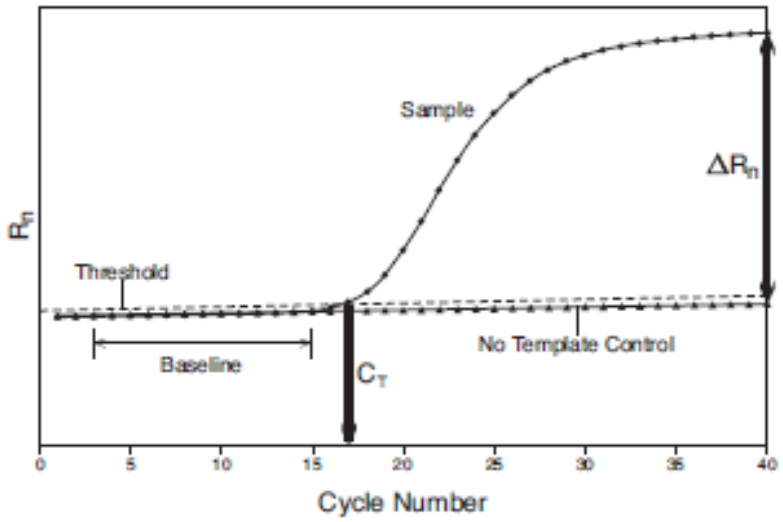


Fig. 19. An example amplification curve from one sample (adapted from www3.appliedbiosystems.com).

In Fig. 19 an example of a single amplification curve can be seen. The amplification curve has regions defined below that are used to determine the relative quantitation (RQ) of mRNA in each sample:

- Normalized reporter (R_n) – The ratio of fluorescence emission intensity from the reporter FAM™dye to the fluorescence emission intensity of the passive reference VIC™ dye.
- Delta R_n (ΔR_n) – The magnitude of the fluorescence signal generated when the probe binds to the target sequence after normalisation and baseline correction ($\Delta R_n = R_n$-baseline).
- Threshold cycle (C_T)- The cycle number at which the fluorescence signal from the target sequence is first detected. This is determined by the point at which the threshold line crosses the amplification plot.
- Threshold- The level used to determine the C_T. It is set within the exponential phase automatically determined by the software or manually set.

To analyse data in this thesis, the baseline was manually adjusted so that the amplification curve began just after the maximum baseline. The threshold was adjusted manually to be in the middle of the exponential phase so an accurate cycle number was recorded as the Ct value. The results were transferred to Excel (Microsoft Office) for analysis. In Excel, the target sequence Ct values were first adjusted for variances between RNA and volume using the ribosomal 18S Ct value (18S Ct – Gene Ct). Then an average of duplicates was taken to determine the difference (delta) in Ct therefore, delta Ct =Average (18S Ct – Gene Ct) (18S Ct – Gene Ct). The expression levels of the target sequence were normalised to the expression levels of the calibrator sample. Therefore, calibrator sample = 0 and gene expression = deltadeltaCt. As this was a log scale, the final fold–expression changes were calculated as: Fold change = 2^(-deltadeltaCt) = $2^{-\Delta\Delta Ct}$

2.4.5 Primer/Probe validation

Primers and probes were designed using the Primer Express® software (AppliedBiosystems) to identify suitable sequences. The following parameters were used for identifying nucleotide sequences:

- Sequence length of approximately 20bp, amplicon size 50-150bases.
- Avoiding areas of four or more guanines.
- Keeping the total G-C content within 30-80%.
- Designing the primers over intron/exon gaps to decrease the possibility of genomic DNA contamination.
- Selecting primers with a melting temperature (T_m) of 58-60°C
- The five nucleotides nearest the 3' end should have no more that two guanines and/or cytosine bases.
- Designing the primers as close to the probe as possible.

The sequences of the primers and probes used for mouse and human genes are listed below in Table 2 and Table 3 respectively.

Table 2. Mouse gene primers and probes for TaqMan QPCR.

Mouse Gene	forward primer	reverse primer	probe
FGF2	GGCGTCCGCGAGAAGA	CCCTTGATAGACA CAACTCCTCTCT	CGACCCACACGTCAAA CTACAACTCCAA
CXCL1	CCGAAGTCATAG CCACACTCAA.	AATTTTCTGAAC CAAGGGAGCTT	TCGCGAGGCTT GCCTTGACCC
IL6	CCACGGCCTTCCC TACTTC	TGCACAACTCTTTTCT CATTTCCA	TCACAGAGGATACCACTC CCAACAGACCTG
VEGF-A	GCAGGCTGCTGTAAC GATGA	CATGATCTGCATGGT GATGTTG	CCCTGGAGTGCGTG CCCACG

Table 3. Human gene primers and probes for TaqMan QPCR

Human Gene	forward primer	reverse primer	probe
FGF2	CCG ACG GCC GAG TTG AC	GAC ACA ACT CCT CTC TCT T	AGA AGA GCG ACC CTC ACA
CXCL1	GTT TTC AAA TGT TCT CCA GTC ATT ATG	CCG CCA GCC TCT ATC ACA GT	TTC TGA GGA GCC TGC AAC ATG CCA
CXCL8	CTGGCCGTGGCTCTCTT	TTAGCACTCCTTGGC AAAACTG	CCTTCCTGATTTCTGCA GCTCTGTGTGAA
VEGF-A	TAC CTC CAC CAT GCC AAG TG	TAG CTG CGCTGA TAG ACA TCC A	ACT TCG TGA TGA TTC TGC CCTCCT CCT T
COX1	TGT TCGGTGTCC AGTTCCAAT A	ACC TTG AAGGAGT CAGGCATG AG	CGC AAC CGC ATT GCC ATG GAG T
COX2	CCT TCC TCC TGT GCC TGA TG	ACA ATC TCA TTT GAA TCA GGA AGC T	TGC CCG ACT CCC TTG GGT GTC
FP	GCA GCT GCG CTT CTT TCA A	CACTGTCATGAAGATTACT GAAAA AAATAC	CAC AAC CTG CCA GAC GGA AAA CCG
EP1	AGA TGG TGG GCC AGC TTG T	GCC ACC AAC ACC AGC ATT G	CAG CAG ATG CAC GAC ACC ACC ATG
EP2	GAC CGC TTACCTGCAGC TGTA C	TGA AGT TGC AGG CGA GCA	CCA CCC TGC TGC TGC TTC TCA TTG TCT
EP3	GAC GGC CAT TCA GCT TAT GG	TTGAAGATCATT TTCAACATCATTATCA	CTG TCG GTC TGC TGG TCT CCG CTC
EP4	ACG CCG CCT ACT CCT ACA TG	AGA GGA CGG TGG CGA GAA T	ACG CGG GCT TCA GCT CCT TCC T
RCAN1	CGCCAAATCCAGACAAGCA	CGCATCTTCCACTTGTTTCCA	TCTCCCCTCCCGCCTC TCCG
ADAMTS1	GGACAGGTGCAAGCT CATCTG	TCTACAACCTTGGGC TGCAAA	CAAGCCAAAGGCATTGG CTACTTCTTCG
18S	CGG CTA CCA CAT CCA AGG AA	GCTGGAATT ACCGCG GCT	TGCTGGCACCAGACT TGCCCTC

New primers and probes were initially validated using serial dilutions of control cDNA from a range of 40-1.25 ng/µl. The mouse specific primers and probes, seen in Table 2, were validated using mouse kidney cDNA whereas human primers and probes, seen in Table 3 were validated using a cDNA of normal endometrium.

As an example, Fig. 20 and Fig. 21 display primer probe validation graphs for ADAMTS1 (Table 3). Firstly, the Ct values for ribosomal 18S and target sequence (ADAMTS1) are plotted in a linear regression graph against the Log values of control cDNA concentration (Fig. 20). The y=mx+c equation of the straight line is displayed on the graph. R^2 should be close to 1.

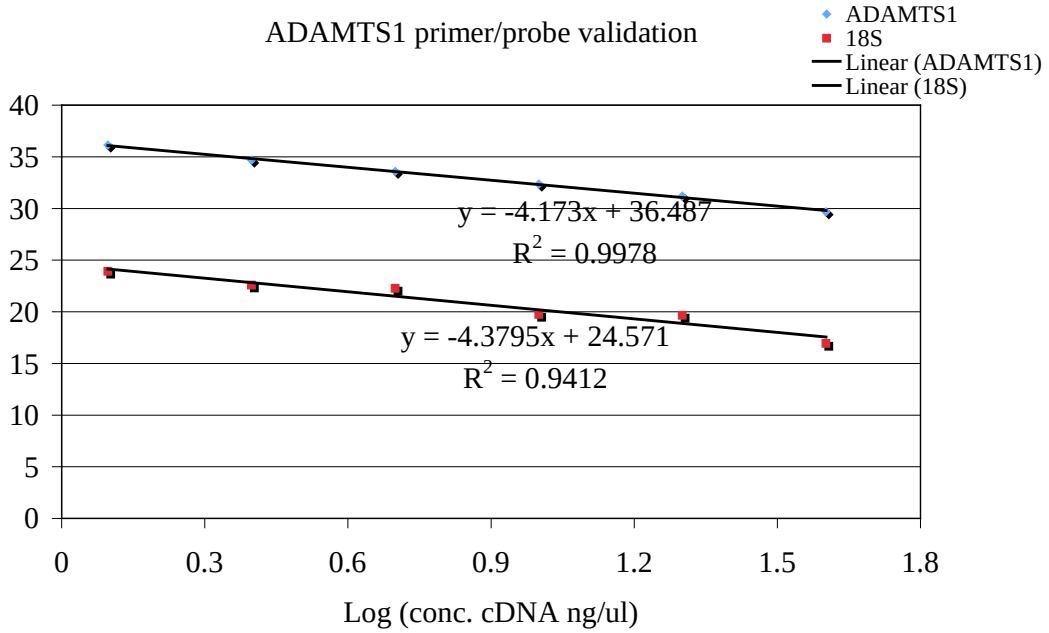


Fig. 20. Graph displaying the linear equations for target gene sequence and 18S control.

Next, as seen in Fig. 21, the deltaCt values for the target gene sequence (ADAMTS1) were plotted against the Log of concentration of cDNA (Fig. 21). The equation of a straight line y=mx+c is displayed on the graph. m, the gradient of the straight line, should be close to 0, the gradient for a horizontal line. Indicating that the 18S and gene of interest are present in the same proportions at each concentration of cDNA therefore, the primers and probes are reliably relaying the quantities of the target sequence.

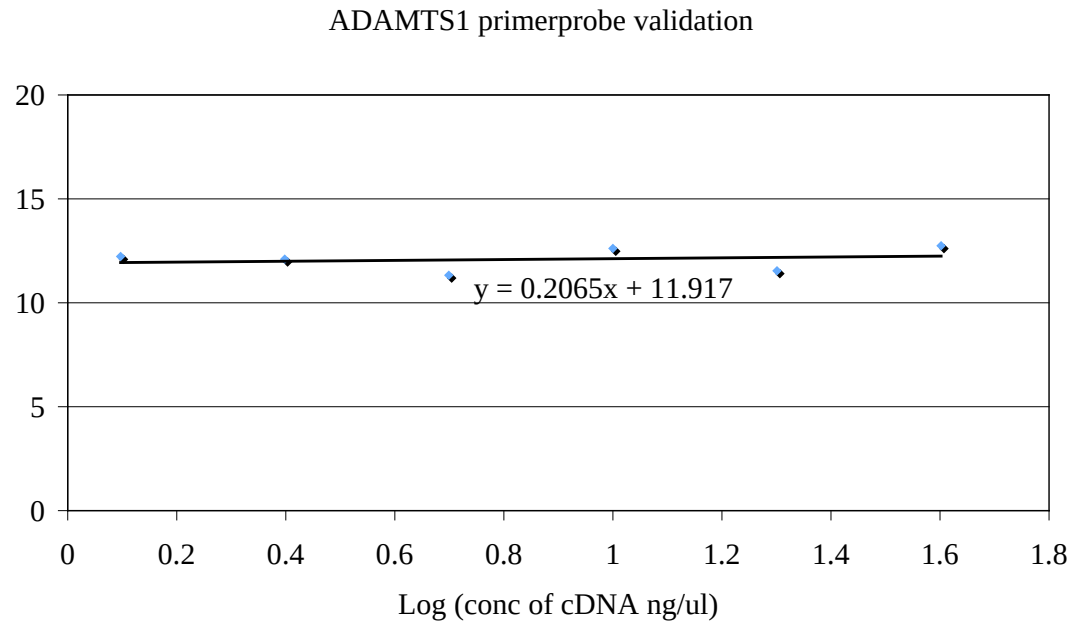


Fig. 21. Graph displaying the linear equation of the deltaCt of the target gene sequence.

2.5 Protein Analysis

2.5.1 Protein extraction from cells

HUVEC were seeded at $2x10^5$ cells per $60mm^2$ dish and left to adhere for 24hrs, at 37^oC in a 5% CO_2 atmosphere, before serum starvation overnight. Growth factor-starved HUVECs were treated with P CM or V CM for 0, 5, 10, 15, 20, 30 mins in the case of a time-course and 10minutes when using a panel of chemical inhibitors. HUVEC were rinsed with ice-cold phosphate-buffered saline and lysed on ice for 20mins with 200µl/dish protein lysis buffer containing inhibitor cocktail mix (see section 2.10).

2.5.2 Protein quantification

Protein concentration was quantified by a protein assay (BioRad, Hercules, CA) following the method of Lowry. The standard curve for total protein concentration in a sample was produced using known dilutions of bovine serum albumin (BSA). Standards were made from serial dilutions of 1mg/ml BSA to concentrations of 400, 300, 200, 100, 75, 50 and 25 µg/ml in water. 25 µl of standards and samples were

added in duplicate to the wells of a 96-well plate. An equal volume of dye reagent (Reagent S + Reagent A, 1:50) was added to each well, followed by 100µl of Reagent B. The colour was allowed to develop for 10minutes after which, the plate was read at 690nm on a colorimetric plate reader.

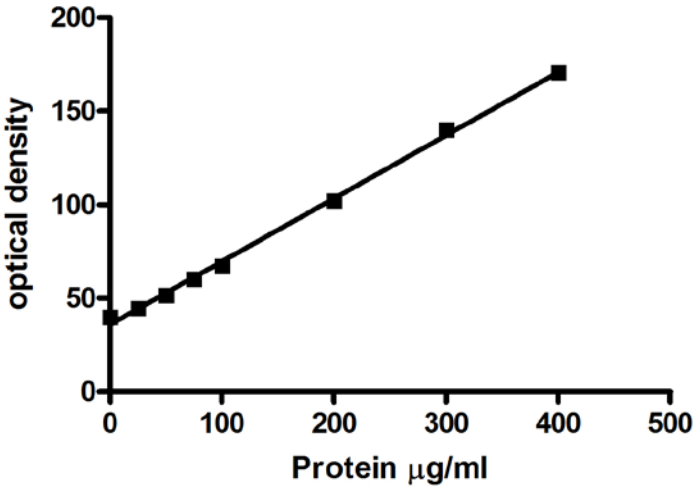


Fig. 22. A typical BSA standard curve for the Bio-Rad protein assay, Lowry method.

An example standard curve is displayed in Fig. 22. The standard curve was constructed as an XY linear regression graph in Prism 5 (GraphPad, San Diego, CA). The optical density value obtained from each sample was used to calculate the unknown protein concentration (µg/ml) from the standard curve.

2.5.3 Acrylamide gel electrophoresis

Approximately 16µg of protein was solubilised in Laemmli buffer (see section) and boiled for 5 min. Protein was resolved using NuPAGE® Novex Bis-Tris Gels. The running buffer was prepared by diluting NuPAGE SDS Running Buffer at a ratio of 1:20 in deionised water. The running buffer contains SDS **(**sodium dodecyl sulphate), to denature and negatively charge the proteins enabling them to migrate through the gel, and EDTA to minimise disulphide bonds which could slow protein migration. MES (pKa = 6.15), and MOPS (pKa = 7.20) SDS running buffer (section 2.10) were used for small or medium molecular weight proteins, respectively, as proteins migrate faster with MES due to its higher acid dissociation constant (pKa) (see www.invitrogen.com for further details). The chambers of the XCell *SureLock* Mini-

Cell (Invitrogen) gel box were filled with buffer to a level required to allow the flow of current. In the first well of the gel, 10µl of coloured standard, SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was loaded. This coloured standard allowed the separation of samples to be monitored. In the next gel wells 25µl of protein sample was loaded. The current was set at 40 mA/gel and samples were allowed to migrate through the gel and separate according to their molecular weight.

2.5.4 Semi-Dry protein transfer

Once the loading buffer reached the bottom of the gel, the gel was removed from its case and the protein transfer module was assembled, see Fig. 23. The semi-dry method of protein transfer was used. Blotting paper was soaked in Transfer buffer (containing methanol, see section 2.10) and a PVDF (Polyvinylidene Difluoride) Immobilon-FL™ membrane (Millipore UK, Watford, UK) was soaked in methanol prior to assembly. The methanol enhances the binding of the protein to the membrane by decreasing the presence of SDS and also helps to stabilise the gel dimensions. Blotting paper was positioned in the transfer module followed by the membrane. The gel was placed on top of the membrane and covered with more blotting paper. The current flowed from the cathode to the anode so that the protein was transferred on to the membrane.

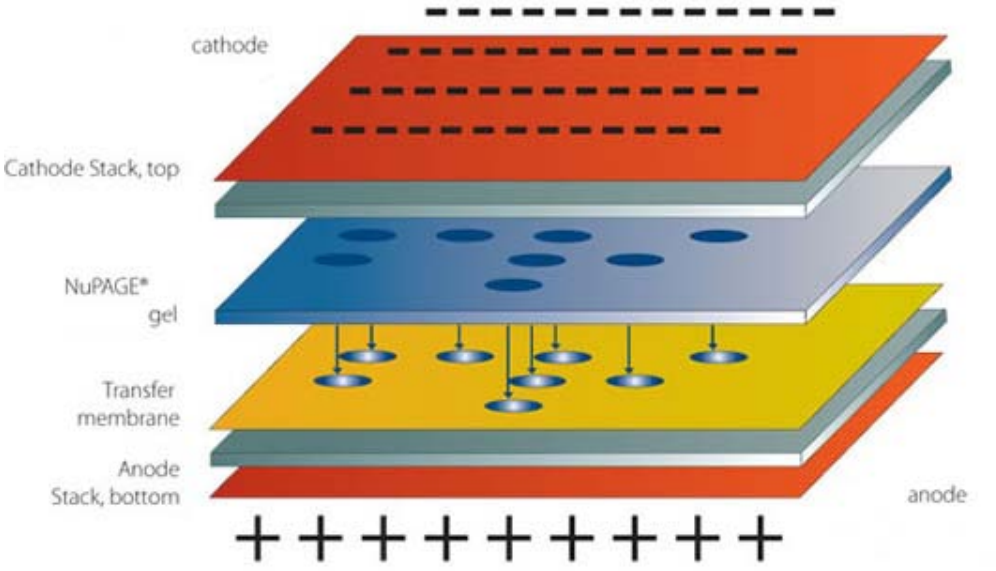


Fig. 23. A standard semi-dry protein transfer module (adapted from http://www.invitrogen.com).

Proteins were allowed to transfer to the membrane for 1.5 hrs at a constant 14volts. The successful transfer of protein was indicated by the presence of the coloured standard on the membrane.

2.5.5 Western blotting

Immunoblots were blocked, to reduce background signals from non-specific antibody binding, in Odyssey Blocking buffer™ (LI-COR Biosciences, Cambridge, UK). Subsequently, immunoblots were incubated overnight with the primary antibodies, listed in Table 4, (diluted 1:1000 in blocking buffer) at 4°C. The following day, blots were washed in PBS and incubated with the secondary antibodies, listed in Table 5, for 60 minutes at room temperature. The IRDye® 800 secondary antibody used is a fluorophore which can be detected with near-infrared (IR) wavelengths (800nm). This longer wavelength helps minimise background when visualising proteins. Simultaneous detection of a control protein was achieved by the use of a fluorophore with an emission spectrum in the 680nm range.

Table 4. Summary of primary antibodies used for Western blotting

Antibody	Source	Dilution
Anti-phospho-p42/p44 ERK	Cell Signaling Technologies	1:1000
Anti-p42/p44 ERK	Cell Signaling Technologies	1:1000
Anti-p85 PI3K	Upstate	1:1000
Anti-phospho p85 PI3K	Autogen bioclear	1:1000

Table 5. Summary of secondary antibodies used for Western blotting

Antibody	Source	Detection	Dilution
Goat anti-mouse	Rockland, USA	800nm	1:10,000
Goat anti-rabbit	Invitrogen, Paisley, UK	680nm	1:5000
Donkey anti-goat	Invitrogen, Paisley, UK	680nm	1:5000
Donkey anti-rabbit	Rockland, USA	800nm	1:5000

2.5.6 Protein expression analysis

The chemiluminescence of immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). Subsequently, protein phosphorylation was calculated by dividing the value obtained from the phosphorylated protein channel (680nm) by the value obtained from total protein channel (800nm) and expressed as fold above vehicle controls.

2.6 Tissue fixation and processing

Tissue samples were fixed overnight in ten percent neutral buffered formalin, pH 7 (NBF, see section 2.10). The NBF facilitates the formation of cross-links which helps keep in position small molecules such as hormones and prevents degradation of proteins whilst preserving their secondary and tertiary structures (see Dako Immunohistochemistry staining guide, 4th Edition, http://www.dakousa.com.) However, it is important not to over fix tissues as this can result in the masking of antigen epitopes. The next day the tissue samples were transferred to 70% EtOH and processed. From the 70% alcohol, the tissue was alcohol rehydrated by incubation in increased concentrations of alcohol, followed by xylene and then samples were embedded in hot paraffin wax on plastic block holders. Sections of tissue samples were cut 5 microns thick with a microtome and mounted on glass slides. Slides were baked at 55°C overnight to ensure the adhesion of the tissue section to the glass.

2.7 Tissue staining and Immunohistochemistry

2.7.1 Haematoxylin and eosin staining

The initial examination of tissue sections was carried out by staining the sections with Harris's haematoxylin (Triangle Biomedical Sciences) and eosin Y (1% aqueous solution mixed with 1% alcohol solution at a ratio of 3:1 - Triangle Biomedical Sciences). Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Following this, the slides were incubated for 5 minutes in haematoxylin, intense staining was decreased by 20 seconds in scotts tap water, and then haematoxylin was 'blued' in water. Two minute incubation in eosin was used to stain the collagen fibres. After optimal staining, sections were dehydrated, incubated

in xylene and mounted with coverslips (VWR, Poole, UK.) using Pertex (Cell Path, Hemel Hempstead, UK).

2.7.2 The priniciples of Immunohistochemistry

Antibodies bind to the epitopes of an antigen through ionic interactions, hydrogen bonds and Van der Waal forces (see Dako Immunohistochemistry staining guide, 4th Edition, http://www.dakousa.com). The affinity of an antibody for an antigen depends on the variable region of its heavy and light chains. Polyclonal and monoclonal antibodies are available for immunohistochemical use. Polyclonal antibody samples contain a range of affinity binding variations due to the multiple epitope binding forms. Polyclonal antibodies are relatively inexpensive and work under a variety of conditions. Monoclonal antibodies are more antigen-specific and their use reduces background, due to non-specific binding, and increases consistency. Affinity-purified antibodies are produced using immobilised antigen to improve antibody specificity. The binding affinity of an antibody affects the incubation time required between the antibody and immobilised tissue antigen.

Below in Fig. 24 is a diagrammatic representation of a primary antibody binding to a target antigen, followed by a secondary antibody binding to the primary antibody.

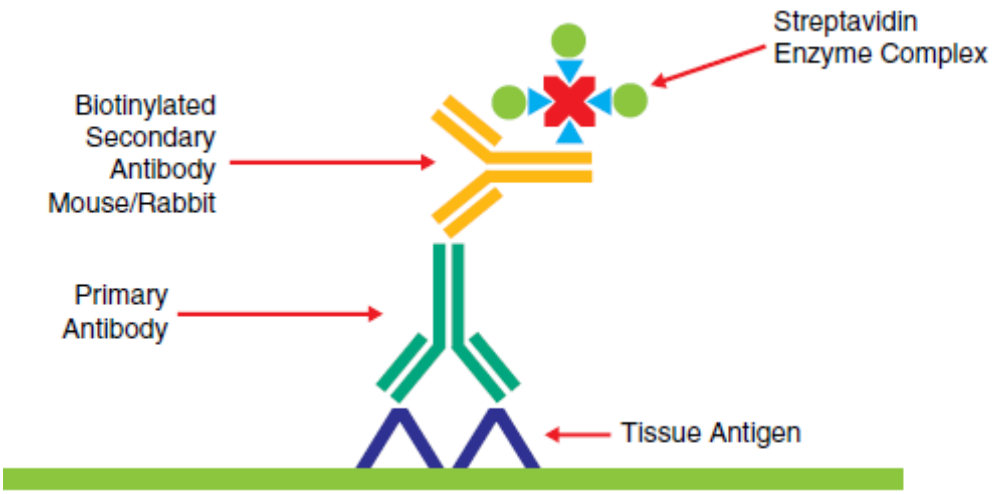


Fig. 24. Target antigen bound to a chain of primary antibody, biotinylated secondary antibody and streptavidin enzyme complex. (adapted from Dako Immunohistochemistry staining guide, 4th Edition, http://www.dakousa.com).

Slides were washed in phosphate buffered saline (PBS) (section 2.10) because streptavidin peroxidase was used as the detection enzyme. Streptavidin horseradish peroxidase (HRP) is a 40 kDa protein that catalyses the oxidation of substrates by hydrogen peroxide. It is most active at a neutral pH and is more stable than the alternative detection enzymes. A commonly used substrate for HRP is 3,3'-diaminobenzidinetrahydrochloride (DAB). When DAB is oxidised by HRP, an insoluble brown coloured product forms which can be visualised under a microscope. Under the correct conditions, the presence of DAB staining will indicate the localisation of the target protein. Whether or not the intensity of DAB staining indicates an increased presence of target protein is contentious because so many factors could affect the DAB intensity.

As an alternative to DAB staining or when coexpression of two or three proteins is required, the method of fluorescent immunohistochemistry can be used. Immunofluorescence (IF) protocols vary depending on the sensitivity of the primary antibody for the target antigen. The simplest IF method involves use of a fluorophore linked secondary antibody. If greater sensitivity is required, a streptavidin linked secondary is applied followed by a species specific fluorophore that will bind to the secondary antibody. Further sensitivity can be added using a tyramide amplification step which lengthens the immunoreactive chain of detection thereby creating a larger fluorescent signal.

2.7.3 Antigen Retrieval

To begin immunohistochemical staining of tissue sections, paraffin embedded sections (slides) were dewaxed and rehydrated following a standard protocol:
5mins in xylene, (x2), 20seconds in 100% ETOH (x2), 20seconds in 95% ETOH, 20seconds in 75% ETOH, wash in tap water. Antigen retrieval was then performed using Citrate buffer (section 2.10) as the retrieval solution. Antigen retrieval involves the incubation of sections in a hot aqueous retrieval solution such as Citrate

buffer. The aim of antigen retrieval is to recover lost immunoreactivity of target antigens which may have been caused by the formalin fixation process. The exact mechanism by which protein immunoreactivity is enhanced by heat is uncertain but it may reverse the cross linking of formalin sensitive antigenic sites (epitopes) (see Dako Immunohistochemistry staining guide, 4th Edition, http://www.dakousa.com). Citrate buffer was diluted to a 10mM concentration in deionised water and brought to the boil in a pressure cooker. Slides were boiled for 5minutes, afterwhich the slides were taken off the heat and left to sit for 20minutes in the hot Citrate buffer.

2.7.4 Methanol peroxide block

A methanol peroxide block was used to minimise background staining that could occur due to endogeneous peroxides in the tissue. Endogenous peroxidises are found in a variety of cells including, red blood cells, monocytes and muscle cells. All slides were blocked in 300mls of methanol with 3% (v/v) hydrogen peroxide at room temperature for 30mins.

2.7.5 Wash buffer

After 30minutes incubation, the slides were washed in water followed by a wash in phosphate buffered saline (PBS) (section 2.10). Generally PBS was used to wash slides after each incubation step. However, in some cases it may cause a higher incidence of nonspecific binding therefore with antibodies that had high levels of background binding such as CD31, Tris Buffered Saline (TBS, section 2.10) was used as an alternative wash buffer. Tween 0.01% was added to TBS and used for the first of three washes as this detergent helps to further decrease non-specific binding. In contrast, PBS helps to reduce auto fluorescence during fluorescent immunohistochemistry therefore it was the wash buffer used during this process.

2.7.6 Serum block

Sections were blocked in 4 parts PBS with 1 part normal serum (NS, e.g rabbit) (Diagnostics Scotland, Carluke, UK) (v/v) and 5% Bovine Serum Albumin (BSA) (Sigma, Poole, Dorset, UK) for at least 30mins at room temp. The normal serum

chosen was usually from the same species that the secondary antibody was raised in to help minimise non-specific binding.

2.7.7 Primary antibodies

After, blocking in serum block, the block was drained off the slide and excess block was wiped from the area around the target antigen. Polyclonal or monoclonal primary antibodies, listed in Table 6, were diluted in serum block (NS/PBS/BSA) and incubated with the immobilised target antigens on glass slides overnight at 4°C. The optimal dilution of primary antibody was pre-determined in trial runs using serial dilutions.

Table 6. Summary of primary antibodies

Antibody	Source	Dilution
ADAMTS1	Abcam, Cambridge, UK	1:100
FP	CaymenChemicalCompany,USA	1:75
COX-2	SantaCruz Biotechnology, USA	1:200
VEGF-A	SamtaCruzBiotechnology,USA	1:200
CXCL1	SantaCruz Biotechnology, USA	1:100
CD31	Santa Cruz Biotechnology,USA	1:350
Goat IgG	Dako	1:200
Rabbit IgG	Dako	1:200

2.7.8 Secondary antibodies

The secondary antibody used to detect each antigen was determined according to the species that the primary antibody was raised in and the detection method. Biotinylated secondary antibodies were used unless otherwise specified (see Table 7). For example, a rabbit anti goat biotinylated, at 1:500 in Normal rabbit serum/TBS/BSA, was applied after the primary goat anti human CD31 (Santa Cruz, sc-1506). Secondary antibodies were incubated for 30 mins at room temperature.

Table 7. Summary of secondary antibodies used for immunohistochemsitry

Host	Target	Conjugate	Source	Dilutions
Rabbit	anti-goat	biotinylated	Dako	1:500
Goat	anti rabbit	biotinylated	Dako	1:500
Swine	anti rabbit	biotinylated	Dako	1:500

2.7.9 Developing, counterstaining and mounting

Prior to DAB staining, streptavidin horseradish peroxidise (HRP) (DAKO, Cambridge, UK) was diluted 1:1000 in PBS (v/v) and was incubated for 30mins with slides. Thereafter, slides were washed three times in PBS for 5 minutes. The DAB substrate (DAKO), 1 drop/ml in diluent, was added to each slide and colour development was monitored microscopically. The reaction was stopped by washing in water. Slides were counterstained with haematoxylin and mounted with coverslips in pertex.

2.7.10 Imaging

High resolution photographs of DAB stained tissue and cells were taken with the Provis microscope (Provis AX70, Olympus America Inc., New York, USA) using Axiovision software (Carl Zeiss GmbH, Hamburg, Germany).

2.8 Secreted protein identification and quantification

2.8.1 Angiogenesis Antibody Array

A Human Angiogenesis Antibody Array I (RayBiotech Inc, Norcross, GA, USA) kit was used to identify angiogenic proteins present in the conditioned medium (section 2.2.4) from Ishikawa cells as per the manufacturer's instructions. Four angiogenesis array membranes were used from the kit. All buffers and reagents were provided in the kit (RayBio, recipes undisclosed). The membranes were incubated in blocking buffer before the addition of samples. Control membranes were treated with 1ml of vehicle-treated WT or FPS cell conditioned medium and the test membranes were

treated with 1ml of $PGF_{2\alpha}$-treated WT or FPS cell conditioned medium (DMEM). Membranes and samples were incubated for 2hrs at room temperature after which, membranes were washed in buffers provided. The cocktail of biotinylated antibodies was added overnight at 4°C. Wash steps were repeated and HRP-streptavidin was incubated with the membrane for 2hrs. After further washing, detection of the membrane bound proteins was determined with detection buffer and exposure to x-ray film which was developed using autoradiography film processing equipment (Compact, Xograph , Tetbury, UK) in a dark room.

2.8.2 Enzyme-linked immunosorbent assays (ELISA)

2.8.2.1 VEGF and FGF2

VEGF and FGF2 protein levels in the conditioned medium were measured using the Human VEGF ELISA Kit (Cat. No. QIA51, Calibiochem) and Basic FGF2 ELISA Kit (Cat No.QIA64, Calibiochem) according to manufacturers instructions. Conditioned medium was diluted 1:10 in assay diluent to detect VEGF and conditioned medium was used undiluted to detect FGF2 levels.

2.8.2.2 CXCL8 and CXCL1

96 well plates (NUNC™, ThermoFisherScientific, Rochester, NY, USA) were coated with 1.8µg/ml capture antibody monoclonal anti-human CXCL8 or CXCL1 (R&D) and incubated overnight at 4°C. The next day, excess contents were expelled and Dry coat (section 2.10) was added for 1hr. Excess dry coat was aspirated and plates were stored at -20°C. Before use, plates were washed with ELISA wash buffer (section 2.10). Recombinant human CXCL8 (20ng/ml) was diluted in ELISA buffer containing tween (section 2.10) to create standard range of 1000-7.8pg/ml. Recombinant CXCL1 (20ng/ml) was diluted in ELISA buffer containing tween to create a standard range of 4000-31.2 pg/ml. 100µl/well of standards and samples were incubated on plate shaker for 3hrs after which plates were washed with wash buffer. Detection antibodies, biotinylated anti-human CXCL8 or CXCL1 antibody (R&D) diluted to 0.1µg/ml in ELISA buffer containing tween, were added to the

plates at 100µl/well and incubated for 1hr on a plate shaker. Plates were washed and 100µl/well Boeringer streptavidin-peroxidase, diluted 1:2000 in ELISA buffer, was added for 20mins. After washing, a solution of Tetramethy bezidine (3mg/ml) in dimethylformamide (TMB) and urea peroxidase (6mg/ml) was added to 100mM sodium acetated buffer pH6. Subsequently, 200µl/well of TMB substrate mixture was incubated for 10minutes until colour developed. The colour reaction was quenched with 50µl/well of 2N sulphuric acid and the plate was read at 450M with a colorimeter.

2.8.2.3 $PGF_{2\alpha}$/PGE_2

HUVECs were seeded at $5x10^4$ cells per 35mm diameter dish and starved overnight. V CM or P CM was added to cells and diluted 1:1 (v/v) with EBM1%. When using chemical inhibitors, cells were preincubated with EBM1% and SU4984, SB225002, NS3984, Indomethacin, SC560 or PD98059 for 30minutes. Subsequently, cells were treated with V CM or P CM in the absence or presence of inhibitors with the addition of 3µg/ml arachidonic acid for 6hrs. After the prescribed time, conditioned medium was collected from the HUVECs and frozen until use. The $PGF_{2\alpha}$ competition ELISA was carried out with diluted conditioned medium (diluted 1:4, 1:10, 1:20, v/v). Briefly, 96 well plates (NUNC™,ThermoFisherScientific) were coated overnight with donkey anti-rabbit (DAR) capture antibody. The next day excess contents were removed and Dry Coat solution added for 1hr. Then excess dry coat was removed and plates were dried before storage for up to seven days at 4°C. The link was prepared, in the laboratory of R.W. Kelly, by ether extraction and purified by reverse phase chromatography using 20mg of synthetic PGE_2 or $PGF_{2\alpha}$, 320µL of dry dimethylformamide, 3µl butylchlororomate and 0.05nM biocytin. Biotin labelled $PGF_{2\alpha}$ link was added at 1 in $1.0x10^6$ and antisera (rabbit IgG; 1mg/ml diluted in PBS with 1% carbonate buffer, pH 9.6) at 1 in 20 000.

For the $PGF_{2\alpha}$ ELISA, samples, standards, link and antiserum were diluted in ELISA buffer without tween (section 2.10) with the highest standard being 5120pg/ml. For the PGE_2 ELISA samples were incubated with a methyloximating (MOX) solution

(Methyloxyamine buffer, pH 5.6, v/v) to stabilise the PGE_2 prior to freezing. The PGE_2 assay was carried out with 25% MOX buffer and ELISA buffer containing tween (section 2.10). Link was diluted 1:1.5 x 10^6 in 0.5M P04 pH8 to balance the MOX solution pH. The antiserum was diluted 1:50000 in ELISA buffer. Standard range of the assay was 5120–10 pg/ml. ELISA plates were washed before use and incubated overnight at 4°C with a solution of antiserum, link, buffer and samples or standards. The following day, plates were washed and 100µl/well streptavidin peroxidase (0.2U/ml) was added for 25mins at RT on a plate shaker. After washing, 200µl/well of TMB substrate mixture was incubated in the wells for 10minutes until colour developed. 50µl/well of 2N sulphuric acid quenched the colour reaction and the plate was read at 450M with a colorimeter. As the prostaglandin ELISA is a competitive ELISA, in which prostaglandin in samples competes with biotin labelled prostaglandin link, less prostaglandin present in the sample media will result in a more intense colour reaction. A standard curve was produced from which the concentrations of $PGF_{2\alpha}$ and PGE_2 in the sample media were determined using AssayZap software (Biosoft, Cambridge, UK).

2.9 Endothelial cell function assays

2.9.1 Network formation assay

The network assay model, seen in Fig. 25, was used to assess endothelial cell differentiation in vitro. Network assays were carried out using 12-well Transwell plates (Corning Costar, Cambridge, UK). Growth Factor (GF)-reduced Matrigel (BD Biosciences,MA, USA) was liquefied overnight on ice at 4^oC. Pipette tips and plates were cooled at -20^oC before use. The upper chambers were coated with 80µl of GF-reduced Matrigel in the absence/presence of SU4984 (20µM), PD98059 (50µM), rapamycin (100ng/ml), wortmannin (200nM), LY294002 (50µM), NS398 (10µM) and SC506 (10µM) and incubated at 37^oC for 30mins to allow thin gel formation. Human umbilical vein endothelial cells were plated onto the gel ($2.5x10^4$cells/well) in EBM 1% (v/v). In the lower chamber V CM or P CM was added (Fig. 25).

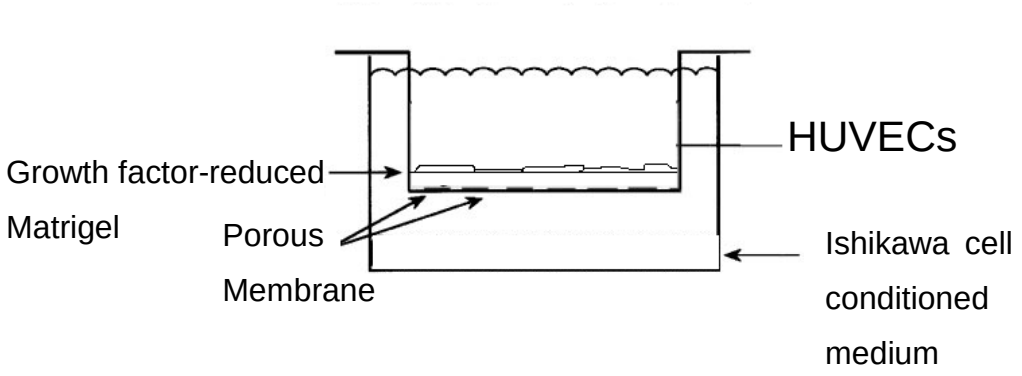


Fig. 25. Dual chamber network assay model.

The use of a dual chamber model for network assays (Fig. 25) was chosen because it reflected the in vivo environment of angiogenesis. Secreted factors from tumour cells travelling through the cell matrix will come into contact with the abluminal surface of endothelial cell which corresponds to the basolateral plasma membrane of cultured endothelial cells (Stolz et al., 1992). Also the distribution of growth factor receptors has been shown to differ in an apical/basal orientation on endothelial cells (Stolz et al., 1992; Vlodavsky et al., 1987). Another advantage to using the transwell chamber is that two different media can be used to optimise the endothelial and epithelial function. DMEM is the preferred medium for Ishikawa cell culture and conditioned medium production, as Ishikawa conditioned medium produced with EBM1% resulted in lower concentrations of growth factors. Vice-versa, culturing HUVECs with serum-free DMEM induced cell death. Pooled conditioned medium was used, as opposed to culturing Ishikawa cells in each of the lower chambers, in order to reduce experimental variability. This differentiation assay is referred to in this thesis as a network assay so as not to be confused with the tubule formation assay, another differentiation assay that takes place over a period of 3-7 days during which lumen-containing tubules of endothelial cells form in a synthetic extracellular matrix containing fibroblasts (Donovan et al., 2001).

Transwell plates were incubated at 37^{o}C in a 5% CO_2 atmosphere for 16hrs. Subsequently, cell networks were fixed with 100% ice cold methanol and stained with haematoxylin.

2.9.1.1 Image analysis

To assess network formation, each well was divided into 5 sections. Hotspots of each section were photographed, 5 photos per well at x10 magnification, using an inverted microscope and camera (Axiovert 200, Carl Zeiss, Germany). The number of network branches was counted blind. Experiments were repeated at least four times. Fold difference was determined by dividing the value obtained from P CM treated cells by the value obtained from V CM treated cells. Data are represented as percentage increase in network formation with V=100% and are presented as mean+SEM.

2.9.2 Proliferation assay

HUVEC in EGM were seeded in 96-well plates at 3000 cells/well. Following attachment, cell medium was replaced with EBM containing 1% FBS (EBM1%;v/v) for 3 hours. Cells were then treated with conditioned medium, diluted 1:1 with EBM1%(v/v), in the absence/presence of SU4984, PD98059, rapamycin, wortmannin, LY294002 or immunoneutralised conditioned medium. Treatments were replaced three times during the 96hr incubation. Proliferation was determined using the CellTitre96AQueous One Solution Proliferation Reagent (Promega, Southampton, UK). As shown in Fig. 26, this reagent contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), which together with an electron coupling agent, phenazine ethosulfate (PES), forms a stable compound. The MTS tetrazolium compound is bioreduced by cells undergoing metabolic activity, resulting in a soluble coloured formazan compound (Fig. 26). This is presumed to be a result of the increased availability of H+ released by the metabolic dehydrogenation of NADPH or NADH to NADP or NAD to respectively. The formation of a soluble coloured formazan compound is directly proportional to the availability of H+ and

consequently, the number of living cells. After a 1-4hr incubation period, the formation of formazan is recorded by measuring the absorbance at 490nm on a 96-well plate reader.

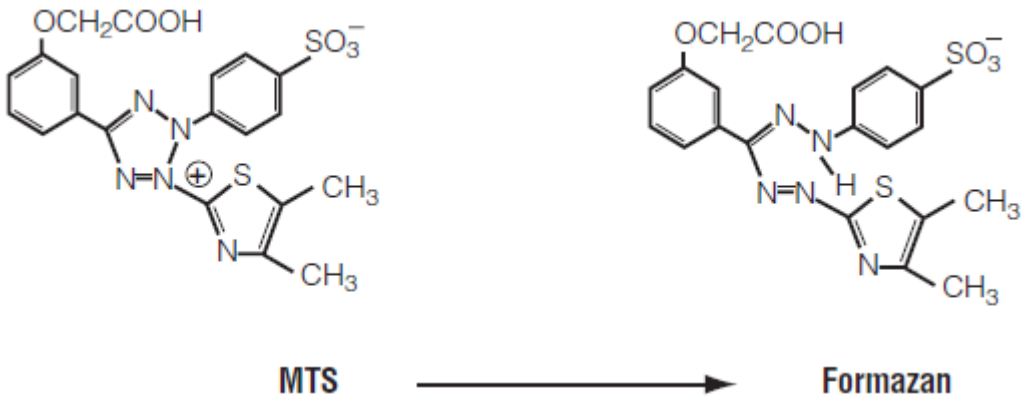


Fig. 26. Structures of MTS and its formazan product (adapted from Promega instructions for use of products G3580, G3581 and G3582).

The experiments were repeated at least three independent times. Fold difference was determined by dividing the absorbance obtained by P CM treated cells by the absorbance obtained by V CM treated cells. Data are represented as percentage increase in proliferation with V =100 % and are presented as mean+SEM. Cell proliferation was assessed under EBM1% and inhibitor alone conditions to verify that basal endothelial cell proliferation was not affected by the addition of inhibitors. This condition was used as a control for normalisation of the results.

2.9.3 Gene manipulation

RNA interference (RNAi) can be mediated by the use of short hairpin RNA (shRNA) or small interfering RNA (siRNA). Transfection of siRNA into cells can be mediated by transfection agents such a polyamine mixture (see section 2.9.3.5). The use of viral vectors to infect cells can enhance the efficiency of RNAi delivery into cells (see sections 2.9.3.2, 2.9.3.3 and 2.9.3.4). Adenoviruses have been used for gene targeting for over a decade. They can be used as replicating or non-replicating vectors (Jaras et al., 2007). Usually, the E1 and E3 regions are used as gene integration sites (Jaras et al., 2007). Using a host cell, a target sequence, for example DNA for a short hairpin RNA in a shuttle plasmid is integrated by homologous

recombination into an adenovirus genomic plasmid (Jaras et al., 2007). Adenoviruses were used at 100 multiplicities of infection (MOI). The recombinant adenovirus remains epichromosomal in the host cell (Jaras et al., 2007) so the adenovirus was re-administered every 48hrs.

Lentiviruses are retroviruses that can infect actively dividing and non-dividing, post-mitotic cells and are therefore commonly used RNAi vectors (Dykxhoorn et al., 2003). The RNAi target-specific insert is made up of a 19-23 nucleotide sequence complementary to the target, followed by a short spacer and the reverse complement of the same target sequence (Sledz and Williams, 2005). Once transcribed by the cells machinery, a 19-23bp stem-loop structure, termed short-hairpin RNA (shRNA), is processed by Dicer into a siRNA that can direct the down-regulation of target gene expression (Sledz and Williams, 2005). Lentiviral vectors stably expressing shRNA against any region of mRNA can be targeted; however, sequence sites for mRNA-binding proteins in the 5′ untranslated region (UTR), 3′ UTR, start codon, or exon – exon boundaries should be avoided (Dykxhoorn et al., 2003). Highly specific RNAi sequences should be used at the lowest concentration possible to reduce non-specific, off-target interactions.

2.9.3.1 Verification of viral transfection

Lentivirus or adenovirus transfection efficiency of HUVECs was examined with lentivirus or adenovirus constructs expressing LacZ. HUVECs were seeded at $1x10^4$cells/well in a 24 well plate. The next day LacZ virus was added to HUVECs at 0, 50 and 100 MOI for adenovirus LacZ and 0, 2, 5 MOI for lentivirus LacZ. After 24hrs, the samples were washed in PBS, fixed for 5 minutes in X-gal fixing solution (section 2.11.1.4), washed again in PBS and stained with an X-gal staining solution (section 2.11.1.4) overnight at 37°C. Treatments were performed in triplicate. Three photos from each well were taken with the Axiovert 200 microscope. The total number of cells and LacZ positive cells were counted. Results were expressed as the percentage of LacZ positive cells/total cells. Transfection

efficiency was approximately 80% at 5MOI and 90% at 100MOI for lentivirus and adenovirus respectively.

2.9.3.2 Adenovirus short hairpin FP construct for FP silencing.

To assess the role of the FP receptor in endothelial cell function, two different short hairpin constructs were used, sh306 and sh478 as well as a control scrambled sequence, sc1311. The viral constructs were produced by Dr. P. Brown using the human FP receptor sequence as a template. The start codon of the FP receptor (NM_000959) was used as a reference and labelled as basepair 1. Thus, the target sequences corresponded to 306 and 478bp downstream and included a scrambled (sc1311) negative control: sh306: 5'-GCTGCGCTTCTTTCAAACA; sh478: 5'-GTGGCCTGGTAATCACTGA; scr: 5'-TTACTCGACGCATGTGCTT. Three $25cm^2$ flasks of HUVECs were seeded at a density of $3x10^5$cells/$25cm^2$flask in EGM (need $9x10^5$cells total). Immediately after, 100MOI of one of the three viruses were added to each flask. The cells were incubated for 24hrs at 37^oC in a 5% CO_2 atmosphere. The next day, the viral infected cells were typsinised, counted and used in the network assay (see section 2.9.1) or in the proliferation assay (see section 2.9.2). HUVECs were treated for 24hrs with scr, sh306 and sh478 viruses and knockdown of the FP receptor was verified with QPCR of FP mRNA expression. As shown in Fig. 27, approximately 50% reduction in FP receptor mRNA expression was observed.

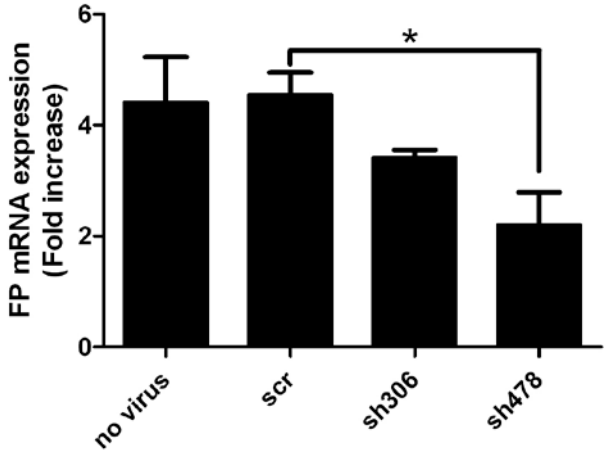


Fig. 27. Confirmation of FP receptor reduction with shRNA. HUVECs were treated for 24hrs with no virus, scrambled virus (scr) and FPshRNA viruses (sh306 or sh478). After

which, FP mRNA expression was assessed by QPCR analysis. Data expressed as fold increase in expression over normal HUVEC control. (* represents statistical significance; P<0.05). Data are represented as mean ± SEM from at least three independent experiments.

2.9.3.3 Adenovirus RCAN1-4 for RCAN1-4 overexpression

The RCAN1-4 adenovirus (Ad-RCAN1), containing the full length RCAN1-4 cDNA (ORIGENE, Rockville, MD), was produced as previously described (Maldonado-Perez et al., 2009). An empty adenovirus was used as the control (Ad-Control). Overexpression of RCAN1-4 was verified and optimized by quantitative RT-PCR (Fig. 28.). Infection of HUVECs with 100MOI of virus increased RCAN1 expression by more that 100% (Fig. 28.). Subsequently, HUVECs were seeded at $4x10^5$cells/25cm^2 flask and 100MOI of adenovirus was added to each flask. Cells were incubated for 24hrs at 37^{o}C in a 5% CO_2 atmosphere, after which the medium was removed, cells were washed and used for network and proliferation assays.

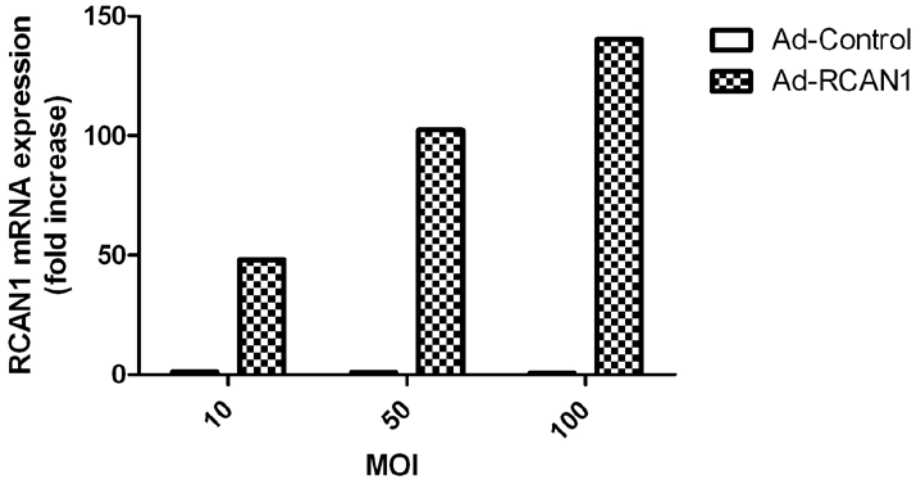


Fig. 28 Adenoviral RCAN1 transfection increases RCAN1 expression in HUVECs. HUVECs were untransfected (0MOI) or transfected with 50MOI and 100MOI of adenovirus construct containing non-target (Ad-Control) or RCAN1-4 cDNA (Ad-RCAN1). RCAN1 mRNA expression was determined by quantitative RT-PCR.

2.9.3.4 Lentivirus RCAN1 for RCAN1 silencing.

The short hairpin RNA (shRNA) lentivirus for RCAN1-4 was obtained from the laboratory of Dr. A.Thompson (Jacksonville, Florida, USA) (Bush et al., 2007).

Flasks, of 25cm^2 seeded with 4x10^5 HUVECs, were transfected with 5MOI of virus for 24 hrs. RCAN1 was expressed at very low basal levels in the absence of stimulation therefore, to verify the efficacy of lv-RCAN1 transfection, lv-Control and lv-RCAN1-4 HUVECs were treated for 3hrs with V CM and P CM. In lv-Control cells, RCAN1 mRNA expression was significantly elevated by P CM treatment compared to V CM treatment (Fig. 29, $P<0.05$). The expression of RCAN1 in lv-RCAN1 cells was significantly decreased after P CM treatment compared to lv-Control cells treated with P CM (Fig. 29, $P<0.05$). This indicates that lv-RCAN1 successfully decreased HUVEC RCAN1 expression. After 24hrs at 37^{o}C in a 5% CO_2 atmosphere, the cells were washed and used in the network and proliferation assays.

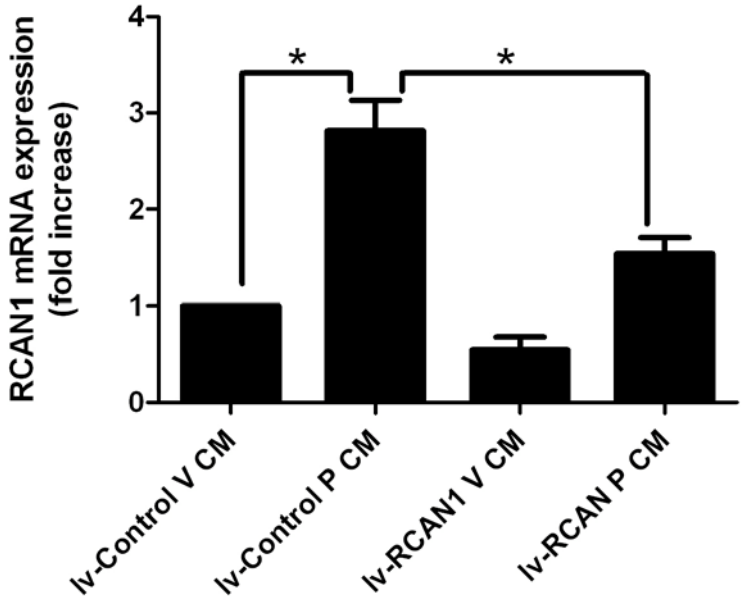


Fig. 29. Lenviral RCAN1 shRNA decreases RCAN1 mRNA expression. HUVECs were transfected with lentivirus containing a control non-target construct (lv-Control) or RCAN1 shRNA (lv-RCAN1) prior to the experiment. Lv-Control and lv-RCAN HUVECs were treated with V CM or P CM for 3hrs and RCAN1 mRNA expression was examined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from at least three independent experiments.

2.9.3.5 siRNA transfection for ADAMTS1 silencing

ADAMTS1 siRNA was used to silence ADAMTS1 expression in HUVECs. ADAMTS1 Stealth siRNA was purchased from Invitrogen. Prior to the start of experiments the concentration of siRNA and transfection agent was optimised. A scrambled non-target sequence of siRNA was used as a control. HUVECs were seeded at $4x10^5$cells/25cm^2 flask. The next day, cells were transfected with 20nM Control siRNA or ADAMTS1siRNA using 5.7µl siPORT Amine Transfection Agent (AppliedBiosystems). As shown in Fig.30, ADAMTS1 siRNA significantly reduced ADAMTS1 mRNA expression by approximately 50% compared to a scramble control siRNA (Fig.30, $P<0.05$). HUVECs were transfected with Control siRNA and ADAMTS1 siRNA for 48hrs at 37^{o}C in a 5% CO_2 atmosphere after which cells were washed and treated for 12hrs with EGM before performing network assays and proliferation assays.

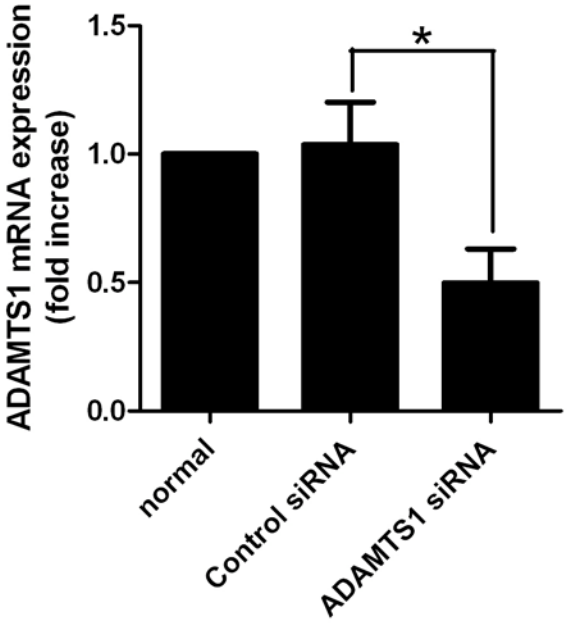


Fig.30. ADAMTS1 siRNA decreases ADAMTS1 mRNA expression. HUVECs were transfected with Control siRNA or ADAMTS1 siRNA for 48hrs prior treatment. ADAMTS1 mRNA expression was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from at least 3 independent experiments.

2.10 In vivo mouse models

2.10.1 In vivo sponge/matrigel mouse model

The in vivo sponge/matrigel mouse model was performed by Dr. Hadoke (Centre for Cardiovascular Science, Edinburgh). Mice were divided into two treatment groups and each participant was given a number from 1-12 therefore Group 1 contained even numbered mice 2, 4, 8, 10 and 12 and Group 2 the odd numbered mice 3, 5, 7, 9 and 11. One set of mice from each group, 6 and 1, was discounted because sponges were absent or too small. Group 1 mice were implanted with matrigel soaked sponges in their left and right sides which were untreated and vehicle treated respectively. Group 2 matrigel sponges were treated with 100nM $PGF_{2\alpha}$ or vehicle in the left and right sides respectively. After 21 days implantation the sponges were excised and divided in two, one half being preserved in RNAlater and the other embedded in paraffin wax for microtome sectioning and immunohistochemical analysis.

2.10.1.1 Assessment of sponge vascularity

Sponge vascularity was examined using paraffin wax embedded sponge sections. Firstly, slide sections were given a code so that vessels were counted blind. Immunohistochemical analysis of CD31/PECAM-1 was chosen to highlight sponge blood vessels as it is a widely used marker for endothelial cells (Donoghue et al., 2007). Once CD31 staining was optimised, a 25-point Chalkey eyepiece graticule (Graticules Ltd, Edenbridge, Kent, UK) at x250 magnification (the graticule covers an area of 0.155 mm^2 at this magnification) was used to quantify vessels (Hague et al., 2002). Hotspots, areas of high vessel density, were identified at a higher magnification before counting began. Blood vessels in three hotspots from each sponge section were counted as previously described (Hague et al., 2002). The graticule was positioned so the maximum number of points touched vessels in one hotspot. The number of dots touching the vessels in each hotspot was then recorded. Individual density was then obtained by taking the mean of three graticule counts.

2.10.2 In vivo tumour xenograft mouse model

The mouse tumour xenograft experiment was performed in the laboratory of Dr Rosenburg (Univ. of Connecticut Medical Centre, USA). FPS or WT cells ($5x10^6$ cells per injection) were subcutaneously injected into the back flanks of adult nude mice. Each day the tumour size was measured none invasively with callipers. After 39 days the resulting tumours were removed for analysis. Half the tumours were preserved in RNAlater for mRNA analysis and half were embedded in paraffin wax for immunohistochemistry analysis.

The tumour xenograft mouse model with CXCL8 immunoneutralisation was performed by Dr Martin Wilson in the Jabbour laboratory. Briefly, a suspension of $5x10^6$ Ishikawa WT or FPS cells in a total volume of 0.2 ml DMEM was injected s.c. into each dorsal flank of CD1-*Foxn1*^{nu} mice (Charles River). Tumours were monitored twice weekly using digital callipers and 100 μg CXCL8 antibody was injected via intra-peritoneal. injection into the mice with FP tumours requiring CXCL8 immunoneutralisation. Control IgG (100μg) was injected into the remaining mice containing WT and FPS xenografts. The animals were maintained under sterile conditions in individually vented cages and all animal care and experimental protocols were approved by the animal ethics committee of the University of Edinburgh and the Home Office of the United Kingdom government. After four weeks, the tumours were removed and divided in two, one half being preserved in RNAlater and the other embedded in paraffin wax for microtome sectioning and immunohistochemical analysis.

2.10.2.1 Assessment of tumour xenograft vascularity

Subsequently, immunohistochemical analysis of CD31 in tumour xenograft sections was carried out as described in section 2.7. Firstly, slide sections were given a code so that vessels were counted blind. Microvascular density was assessed by counting the number of vessels per mm^2 in the outer area of the tumour xenografts, containing the fibroblast layer, using a Nikon Eclipse E800 microscope fitted with an automatic

stage (Prior Scientific Instruments Ltd.) and video camera (HV-C20; Hitachi). Data were analyzed with Image-ProPlus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics). Vessel diameter was measured (μm) and vessels were separated into small (<50μm), medium (50-200μm) and large (>200μm) groups.

2.11 Statistics

In accordance with previous published data on similar studies with the same cell populations and cell treatments, normal distriubution of data was assumed, i.e. that the variance within each population was equal (Sales et al., 2009; Sales et al., 2005; Sales et al., 2004c). Therefore, unless otherwise stated in chapters 3 to 6, statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test using Prism 5.0 (Graph Pad, San Diego, CA). A *p*-value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

For all cell culture experiments a minimum of three independent experiments, preformed on three individual days, was assumed large enough to ensure that the power (probability of correctly rejecting a false null hypothesis) was satisfactorally high. On occasion, where stated, the number of experimental repeats was increased to ensure minimisation of Type II errors (false null hypothesis failed to be rejected). For all experiments the null hypothsis was that the treatment would have no effect on the population compared with the reference population.

2.11.1 Experimental design

2.11.1.1 Chapter 3

The Ishikawa cell timecourse experiment (Fig. 33A) was performed in duplicate, three independent times (n=3). Fold difference was determined by dividing the value obtained from $PGF_{2\alpha}$ treated cells by the value obtained from vehicle treated cells at each individual timepoint. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

The Ishikawa cell experiment with inhibitors (Fig. 33B) was performed using one well per treatment, three independent times for each inhibitor (n=3). Data sets from multiple treatments were combined for graphical display. Therefore the vehicle and $PGF_{2\alpha}$ bars represent nine independent experiments (n=9). Fold difference was determined by dividing the value obtained from $PGF_{2\alpha}$ treated cells by the value obtained from vehicle treated control cells. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

The Ishikawa cell experiment with RCAN1 adenovirus (Fig. 34A) was performed using one well per treatment, four independent times (n=4). Ishikawa cell experiment with RCAN1 lentivirus (Fig. 34B) was performed using one well per treatment, four independent times (n=4). Fold difference was determined by dividing the value obtained from vehicle or $PGF_{2\alpha}$ treated cells by the value obtained from normal endometrial control HNJ124. Data are presented as mean ± SEM. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

2.11.1.2 Chapter 4 and Chapter 5

All network formation assays were performed in duplicate (two wells per treatment) four independent times (n=4). Data were blinded before quantification of network branches. Fold difference was determined by dividing the value obtained from P CM-treated cells by the value obtained from V-CM treated cells. Data were transformed to percentage increase in network formation with V CM =100% and are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

All proliferation assays were performed in quadruplicate (four wells per treatment) four independent times (n=4). Fold difference was determined by dividing the value obtained from P CM-treated cells by the value obtained from V CM-treated cells. Data were transformed to percentage increase in proliferation with V CM =100% and

are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

Western blot experiments were performed using one well per treatment, four independent times (n=4). For Western blot semi-quantitative analysis, ERK1/2 phosphorylation was calculated by dividing the value obtained from the phosphorylated ERK1/2 channel (700nm) by the value obtained from total ERK1/2 channel (800nm) and expressed as fold above vehicle controls. Data are presented as mean ± SEM. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

To perform endothelial timecouse experiments, HUVECs were seeded sub-confluently ($5x10^4$ cells per 35mm diameter well). The experiment was performed using one well per treatment, five independent times (n=5). Fold difference was determined by dividing the value obtained from P CM-treated cells by the value obtained from V CM-treated cells, at each individual timepoint. Data are presented as mean ± SEM. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

Experiments to measure $PGF_{2\alpha}$ and PGE_2 secretion from HUVECs (Fig. 49 and Fig. 50) were performed using single well treatments three independent times (n=3). ELISA data are presented as mean ± SEM in pg/ml. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

Experiments using FP shRNA adenovirus-treated HUVECs (Fig. 59) were performed five independent times (n=5) in duplicate or in quadruplicate for the network assays or proliferation assays respectively. Data were blinded before quantification of network branches. Fold difference was determined by dividing the value obtained from adenovirus (scr, sh306 or sh478)-treated cells by the value obtained from adenovirus control cells (scr) treated with V CM (scr V CM). Data were transformed to percentage increase in network formation or proliferation with scr V CM =100%

and are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

2.11.1.3 Chapter 6

Experiments using ADAMTS1 siRNA-treated HUVECs (Fig. 68) were performed four independent times (n=4) in duplicate or in quadruplicate for the network assays or proliferation assays respectively. Data were blinded before quantification of network branches. Fold difference was determined by dividing the value obtained from treated cells by the value obtained from control siRNA cells treated with V CM (control siRNA V CM). Data were transformed to percentage increase in network formation or proliferation with control siRNA V CM =100% and are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

Experiments using RCAN1 adenovirus treated HUVECs (Fig. 70 and Fig. 72) were performed four independent times (n=4) in duplicate or in quadruplicate for the network assays or proliferation assays respectively. Data were blinded before quantification of network branches. Fold difference was determined by dividing the value obtained from treated cells by the value obtained from Adnenovirus control cells treated with V CM (Ad-control V CM). Data were transformed to percentage increase in network formation or proliferation with Ad-control V CM =100% and are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

Time-course experiments with RCAN1 adenovirus-treated HUVECs (Fig. 71 and Fig. 73) were performed using one well per treatment, five independent times (n=5). Fold difference was determined by dividing the value obtained from treated cells by the value obtained from an untreated HUVEC control sample. Data are presented as mean ± SEM. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

Experiments using RCAN1 lentivirus-treated HUVECs (Fig. 74A and B) were performed four independent times (n=4) in duplicate or in quadruplicate for the network assays or proliferation assays respectively. Fold difference was determined by dividing the value obtained from treated cells by the value obtained from lentivirus control cells treated with V CM (lv-Control V CM). Data were transformed to percentage increase in network formation or proliferation with lv-Control V CM =100% and are presented as mean ± SEM. Statistical significance was assessed on untransformed data with one-way ANOVA and Dunnett's post hoc test.

Timecourse experiments performed with RCAN1 lentivirus-treated HUVECs (Fig. 75 and Fig. 76) were performed using one well per treatment, four independent times (n=4). Fold difference was determined by dividing the value obtained from treated cells by the value obtained from lentivirus control cells treated with V CM (lv-Control V CM). Data are presented as mean ± SEM. Statistical significance was assessed on data with one-way ANOVA and Dunnett's post hoc test.

2.11.1.4 Chapter 7

In chapter 7, where an experiment consisted of only two data sets, each bar represented 10 independent samples (n=10) (Fig. 82-85). Data are expressed as fold difference over control mRNA. Control mRNA was either normal endometrial sample HNJ124 or mouse kidney sample for the analysis of human mRNA and mouse mRNA expression, respectively. Statistical significance was assessed on untransformed data with a Student's unpaired t-test using Prism (Graph Pad, San Diego, CA). A *p*-value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

2.12 Commonly Used solutions

2.12.1.1 Protein Analysis

NP40 Lysis Buffer

- 150mM NaCl
- 10mM EDTA
- 50mM Tris pH7.4
- 0.6% NP40
- 0.05% sodium deoxycholate
- 10% Glycerol
- Make up to 500mls with dH_2O

1 Complete Mini Protease Inhibitor Cocktail Tablet (Roche, Mannheim, Germany) was added to 10mls of NP40 lysis buffer.

Lammeli Buffer (loading buffer)

- 125 mm Tris-HCl (pH 6.8)
- 4% sodium dodecyl sulphate
- 5% 2-mercaptoethanol
- 20% glycerol
- 0.05% bromophenol blue

20x NuPAGE MOPS

- 50mM MOPS (104.6g)
- 50mM TRIS base (60.6g)
- 0.1% SDS (10g)
- 1mM EDTA (3g)
- Make up to 500ml with H_20

20x NuPAGE MES

- 50mM MES (97.6g)
- 50mM TRIS base (60.6g)
- 0.1% SDS (10g)
- 1mM EDTA (3g)
- Make up to 500ml with H_20

2.12.1.2 Immunhistochemistry

Transfer buffer

- 14.4g Glycine
- 3g Tris
- 20% Methanol
- Make up to 1 litre with dH_2O

Tris Buffered Saline (TBS)

- 60.5g Tris
- 87.6g NaCl
- 300mls HCL
- pH was adjusted to 7.4 using concentrated ethanol

Neutral Buffered Formalin (NBF)

- 100 mL Formalin (40 percent formaldehyde)
- 6.5 g Dibasic sodium phosphate
- 4.0 g Monobasic sodium phosphate
- 900 mL Distilled water
- pH was adjusted to 7

Citrate Buffer, pH6 (0.1M)

- Citric acid monohydrate (42.02g)
- Distilled water (900ml)
- pH to 5.5 with concentrated NaOH
- Make up to 2L and pH to 6

2.12.1.3 ELISA reagents

Preservatives

- 200mg/ml 2-Methylisothiazolone
- 200mg/ml Bromonitro Dioxane

In dimethyl sulfoxide (DMSO) 1:1 (V/V)

Dry Coat

- 2% Polyvinyl pyrollidone
- 5mg/ml BSA
- 1ml preservatives
- 5mM EDTA
- Tris 50mM
- Make up to 1litre with dH_2O

Wash buffer (x20)

- 20mls Tween 20
- 360g NaCL
- 48.4g Tris
- 200mls dH_2O
- pH to 7-7.5

ELISA buffer (x1)

- 100mM Tris
- 2mg/ml BSA
- 9g NaCL
- 2mM EDTA
- 300ul phenol red solution
- 1000mls dH_2O
- pH to 7.2

Add 1.5mls of 20% Tween 20 solution to 500mls for ELISA buffer with Tween.

2.12.1.4 X-gal staining

Fixing solution

- 2 % Formaldehyde
- 0.2% Glutaraldehyde

In PBS

Staining solution

- 5mM $K_3Fe(CN)_6$
- 5mM $K_4Fe(CN)_7$
- 2mM $MgCl_2$
- 1mg/ml X-gal

In PBS

3 The expression and regulation of ADAMTS1 by $PGF_{2\alpha}$-FP signalling in endometrial adenocarcinoma.

3.1 Abstract

ADAMTS1 is an antiangiogenic protein responsible for the degradation of extracellular matrix. This chapter demonstrates that ADAMTS1 mRNA expression is elevated in the mid-late secretory stage of normal endometrium and provides evidence to suggest that ADAMTS1 is elevated in poorly differentiated endometrial adenocarcinoma. Immunohistochemical analysis showed ADAMTS1 is expressed in the glandular epithelial cells and vasculature of the normal endometrium and endometrial adenocarcinoma. In addition, using the endometrial cancer cell line, Ishikawa, stably expressing the FP receptor to levels seen in endometrial cancer (FPS cells), ADAMTS1 expression was found to be regulated by $PGF_{2\alpha}$ signalling in a time dependent manner via a pathway involving the FP receptor, EGFR, calmodulin and NFAT. However, this regulation of ADAMTS1 was not mediated by the induction of regulator of calcineurin (RCAN1), a negative regulator of NFAT signalling. These data suggest ADAMTS1 may play a role in the regulation of vascular function in endometrial adenocarcinoma.

3.2 Introduction

Endometrial adenocarcinoma, originating from the glandular epithelial cells of the uterine endometrial lining, is one of the most prevalent cancers amongst women in the Western world (Jemal et al., 2008a; Westlake and Cooper, 2008). It is a disease which particularly occurs in post menopausal women and recent evidence suggests that mutations in oncogene expression may play a role in the aetiology of the disease (Doll et al., 2008). Data generated by the Jabbour laboratory and others have ascertained a role for the cyclooxygenase (COX)-prostaglandin (PG) axis in the regulation of endometrial adenocarcinomas by increasing cell proliferation and the secretion of angiogenic growth factors (Jabbour et al., 2006b; Tong et al., 2000). This is similar to other cancers where over-expression of COX enzymes and biosynthesis of prostaglandins has been shown to promote cellular proliferation

(Tsuji et al., 1996), inhibit apoptosis (Tsujii and DuBois, 1995) and enhance angiogenesis (Tsujii et al., 1998).

A microarray analysis of an endometrial adenocarcinoma cell line, stably expressing the FP receptor to levels seen in cancer (FPS cells), identified many possible targets of $PGF_{2\alpha}$-FP signalling including angiogenic genes FGF2, VEGF-A, CXCL1 and CXCL8. The upregulation of these angiogenic genes, along with their protein secretion, was subsequently confirmed by in vitro investigations using FPS cells treated with $PGF_{2\alpha}$ (Sales et al., 2007; Sales et al., 2005). In addition to the angiogenic genes upregulated, the antiangiogenic factor known as a disintegrin and metalloprotease with a thrombospondin repeat (ADAMTS1) was also found to be upregulated.

Although, the expression and regulation of ADAMTS1 in endometrial adenocarcinoma has not been studied, in the female reproductive system, ADAMTS1 is essential for ovarian vascular medullary formation as ADAMTS1 null mice exhibit severely dysplastic or disorganised vascular networks (Shozu et al., 2005). ADAMTS1 null mice are also infertile in part due to their impaired ovulation (Mittaz et al., 2004). ADAMTS1 expression has been found in the normal and decidual endometrium. Ng et al. showed that ADAMTS1 expression was restricted to stromal cells surrounding the spiral arteries in the secretory endometrium and glandular epithelial cells (Ng et al., 2006). They provided evidence to suggest that ADAMTS1 is involved in cytokine regulation of endometrial decidualisation (Ng et al., 2006). Research by Wen et al. also showed that the expression of ADAMTS1 by endometrial stromal cells is under the control of gonadal steroids DHT and progesterone (Wen et al., 2006). Henceforth, this chapter investigates the expression and localisation of ADAMTS1 in endometrial adenocarcinoma. In addition, the signal transduction pathway regulating ADAMTS1 expression was investigated using an in vitro model system of Ishikawa endometrial epithelial cells stably expressing the FP receptor to levels seen in endometrial cancer (FPS cells),.

3.3 Results

3.3.1 The expression of ADAMTS1 in normal endometrium and endometrial adenocarcinoma.

To evaluate the expression of ADAMTS1 in endometrial adenocarcinoma compared to its expression across the normal endometrial cycle quantitative RT-PCR was performed. 10 patient samples from each phase of the normal endometrial cycle, proliferative, early secretory, mid-late secretory and each stage of endometrial adenocarcinoma, well differentiated (well), moderately differentiated (mod) and poorly differentiated (poor), were examined for ADAMTS1 mRNA expression (Fig. 31). The levels of ADAMTS1 were dramatically increased in the mid-late secretory samples compared to samples from the proliferative phase of the endometrial cyclye (Fig. 31, $P<0.05$). In addition, the levels of ADAMTS1 in poorly differentiated adenocarcinoma were significantly increased compared to samples taken from the proliferative phase of the endometrial cycle (Fig. 31, $P<0.05$).

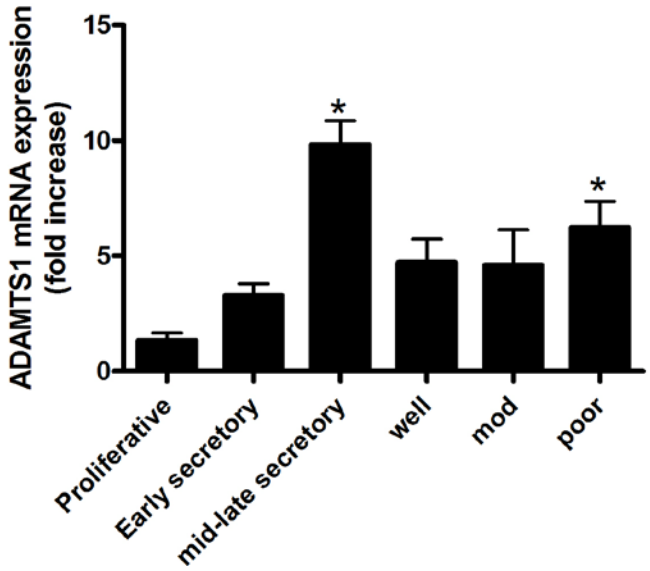


Fig. 31. ADAMTS1 mRNA expression in normal endometrium and endometrial adenocarcinoma. RNA was extracted from 10 patient biopsies from proliferative, early secretory, mid-late secretory normal endometrial samples and well, moderately (mod) and poorly (poor) differentiated endometrial adenocarcinoma samples. ADAMTS1 mRNA expression was analysed by quantitative RT-PCR analysis. (* represents statistical significance compared to proliferative bar; $P<0.05$). Data are represented as mean ± SEM from 10 samples (n=10).

No difference was found between the proliferative phase samples and early secretory phase, well or moderately differentiated adenocarcinoma samples (Fig. 31). Since ADAMTS1 is elevated in poorly differentiated samples, it may play a role in the progression of endometrial adenocarcinoma.

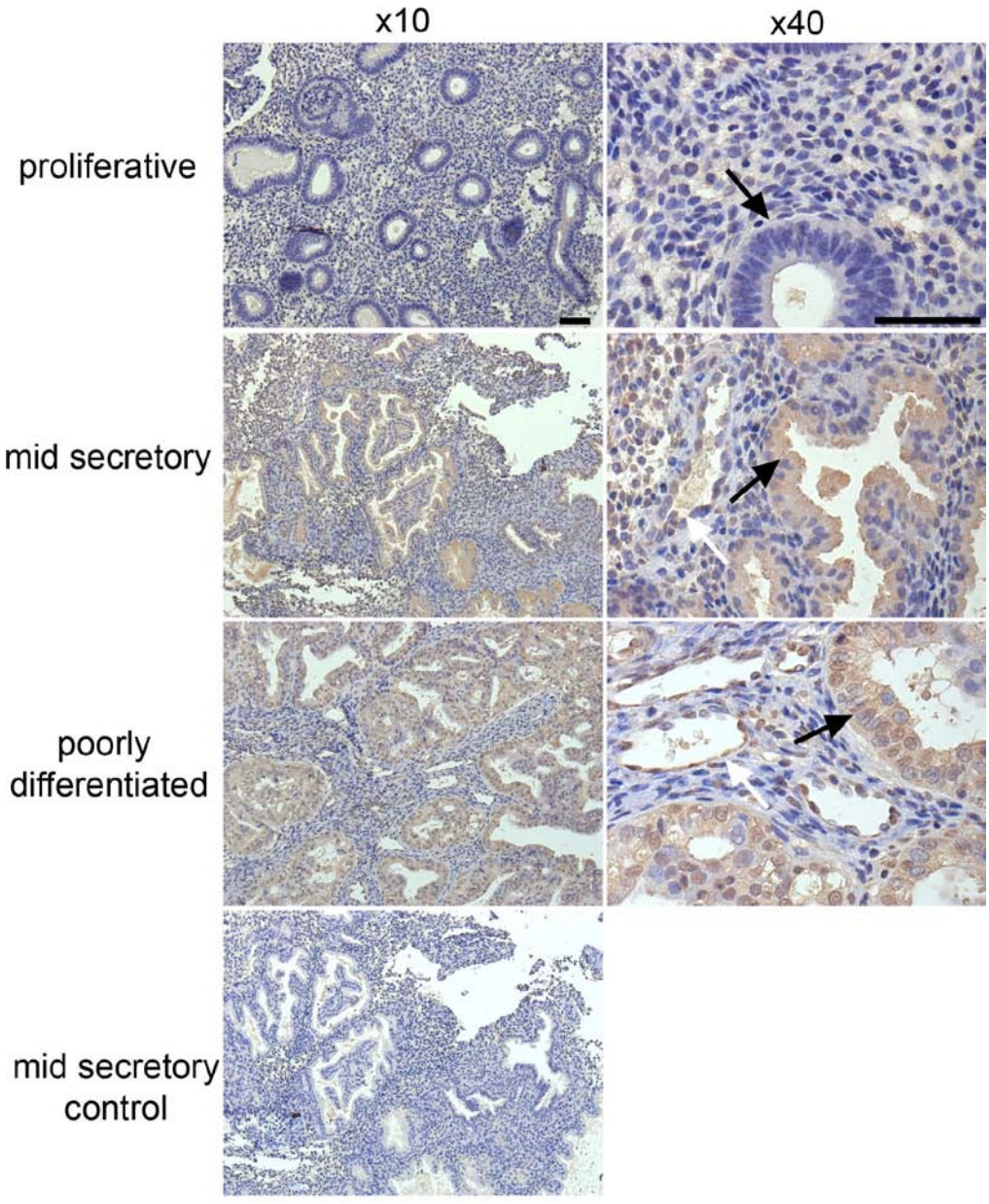


Fig.32. Immunohistochemistry of ADAMTS1 in mid secretory normal endometrium and poorly differentiated endometrial adenocarcinoma samples. The expression of ADAMTS1 in samples from proliferative normal endometrium, mid secretory normal endometrium and poorly differentiated endometrial adenocarcinoma was immunolocalised with ADAMTS1 antibody and DAB, as described in section 2.7. A mid secretory section with rabbit IgG was used as a negative control. White arrows indicate blood vessels. Black arrows indicate glandular epithelial cell layer. Black bars indicate 50μm.

Immunohistochemical analysis of ADAMTS1 localisation in mid secretory normal endometrium samples suggests ADAMTS1 protein is present in both glandular epithelial cells (Fig.32, black arrow) and vascular cells lining blood vessels (Fig.32, white arrow). Similarly, sections of poorly differentiated endometrial adenocarcinoma were stained with ADAMTS1 antibody and prominent staining was observed in the glandular epithelial cells (Fig.32, black arrow) and vascular cells (Fig.32, white arrow).

3.3.2 ADAMTS1 expression and regulation by $PGF_{2\alpha}$-FP signalling in FPS Ishikawa cells.

Since ADAMTS1 and FP receptor expression (Sales et al., 2007) are localised to glandular and vascular compartments and since ADAMTS1 was shown to be a target for FP receptor signalling by microarray analysis, the signalling pathway regulating ADAMTS1 was investigated (Fig. 33). A timecourse of FPS Ishikawa cells treated with 100nM $PGF_{2\alpha}$ for 2 to 72 hrs revealed that ADAMTS1 mRNA expression was significantly upregulated from 4 to 16hrs of $PGF_{2\alpha}$ treatment compared to vehicle treatment (Fig. 33A, $P<0.05$). ADAMTS1 mRNA expression induced by $PGF_{2\alpha}$ was maximal at 6-8hrs (Fig. 33A). Consequently, the signalling pathway regulating the expression of ADAMTS1 after 8hrs of treatment was investigated using a panel of small molecule chemical inhibitors (Fig. 33B). FPS cells were treated with vehicle or $PGF_{2\alpha}$ with or without the addition of AL8810 (FP antagonist), YM-254890 (Gq inhibitor), AG1478 (EGFR inhibitor), INCA-6 (NFAT inhibitor), PP2 (c-Src inhibitor), U73122 (PLC inhibitor), JNK-1 inhibitor, W7 (calmodulin inhibitor), BAPTA (Ca chelator), CsA (calcineurin inhibitor) and PD98059 (MEK inhibitor) (Fig. 33B). ADAMTS1 mRNA expression was inhibited by AL8810, YM-254890, AG1478, INCA6 and BAPTA (Fig. 33B, $P<0.05$). However, ADAMTS1 expression was not affection by the addition of PP2, U73122, JNK-1 inhibitor, BAPTA, CsA or PD98059 (Fig. 33B). This indicates that in FPS cells, the upregulation of ADAMTS1 involves $PGF_{2\alpha}$-FP signalling to EGFR, calmodulin and NFAT.

However, the fact that neither CsA nor BAPTA had an effect on ADAMTS1 mRNA leaves the role of Ca^{2+} and calcineurin in this pathway unclear (Fig. 33B).

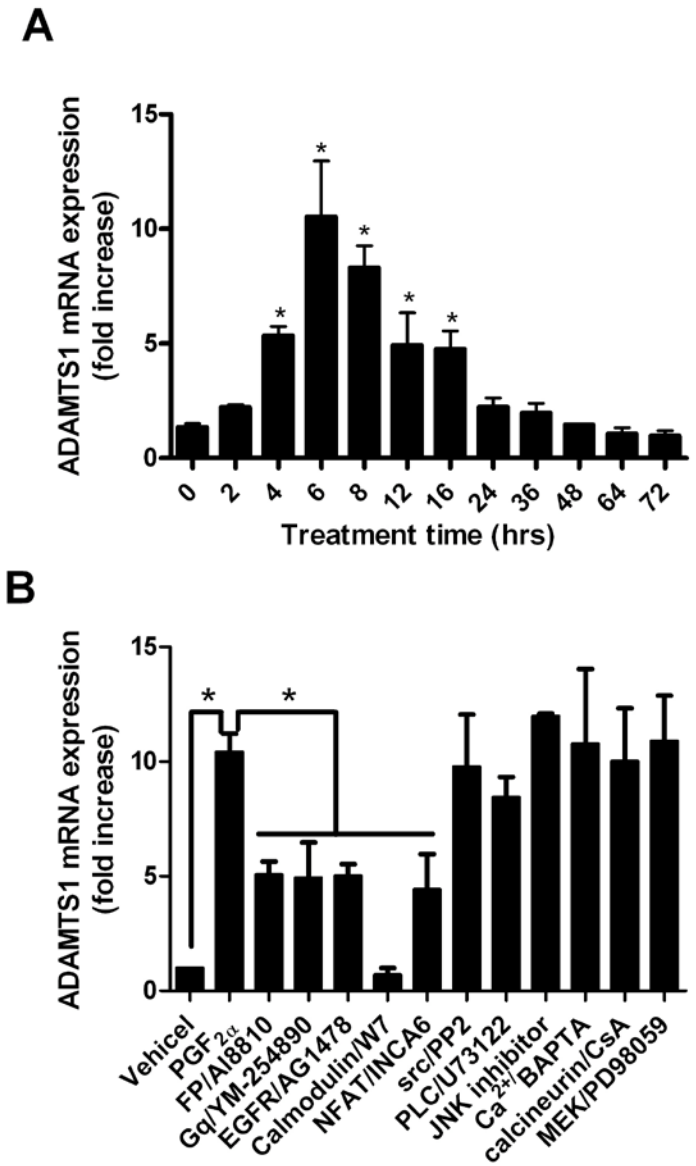


Fig. 33. ADAMTS1 mRNA expression stimulated by $PGF_{2\alpha}$ in FPS cells. A, FPS Ishikawa cells were treated with vehicle or $PGF_{2\alpha}$ (100nM) for 0-72hrs and B, FPS Ishikawa cells were treated for 8hrs with vehicle or $PGF_{2\alpha}$ in the absence/presence of AL8810 (FPantagonist), YM-254890 (Gq inhibitor), AG1478 (EGFR inhibitor), INCA-6 (NFAT inhibitor), PP2 (c-Src inhibitor), U73122 (PLC inhibitor), JNK-1 inhibitor, W7 (calmodulin inhibitor), BAPTA (Ca chelator), CsA (calcineurin inhibitor) and PD98059 (MEK inhibitor) and mRNA expression of ADAMTS1 was determined by quantitative RT-PCR.(* represents statistical significance; $P<0.05$). A, Data are represented as mean ± SEM from 3 independent experiments. B, Data are represented as mean ± SEM from 9 (vehicle and $PGF_{2\alpha}$ bars, n=9) and 3 (each inhibitor bar, n=3) independent experiments respectively.

3.3.3 The role of RCAN1 in regulation of ADAMTS1 expression in FPS cells.

Sales et al. found that CXCL8 upregulation by NFAT was negatively regulated by a negative regulator of calcineurin, RCAN1-4 (Sales, 2009). Therefore, to investigate if RCAN1-4 could regulate NFAT-induced ADAMTS1 expression in FPS Ishikawa cells, cells were infected with control adenovirus (Ad-Control) or adenovirus containing the full length RCAN1-4 cDNA (Ad-RCAN1) (Fig. 34A), as described and performed by Sales et al. (2009). Ad-Control FPS cells treated with $PGF_{2\alpha}$ (100nM) showed significantly higher ADAMTS1 expression compared with Ad-Control FPS cells treated with vehicle (Fig. 34A, $P<0.05$). Similarly, Ad-RCAN1 cells treated with $PGF_{2\alpha}$ showed significantly higher ADAMTS1 expression compared with Ad-RCAN1 FPS cells treated with vehicle. There was no significant difference between the expressions of ADAMTS1 in Ad-RCAN1 cells treated with $PGF_{2\alpha}$ compared to Ad-Control cells treated with $PGF_{2\alpha}$ (Fig. 34A). These data suggest that ADAMTS1 is not regulated by $PGF_{2\alpha}$ via the induction of RCAN1, as observed for proangiogenic factors such as CXCL8 (Sales et al, 2009).

To elucidate the effect that decreasing RCAN1-4 expression in FPS cells would have on ADAMTS1 mRNA expression, RCAN1-4 expression was inhibited in FPS cells using lentivirus containing shRNA against RCAN1 (lv-RCAN1), as described and performed by Sales et al. (2009), and compared with a non-target control lentivirus (lv-Control) (Fig. 34B). lv-Control or lv-RCAN1 FPS cells were treated with vehicle or $PGF_{2\alpha}$ for 12hrs. No significant change in ADAMTS1 expression was observed between lv-RCAN1 FPS cells treated with $PGF_{2\alpha}$ and lv-Control FPS cells treated with $PGF_{2\alpha}$ (Fig. 34B). However, the basal levels of ADAMTS1 were significantly increased in lv-RCAN1 FPS cells treated with vehicle compared to lv-Control cells treated with vehicle (Fig. 34B, $P<0.05$).

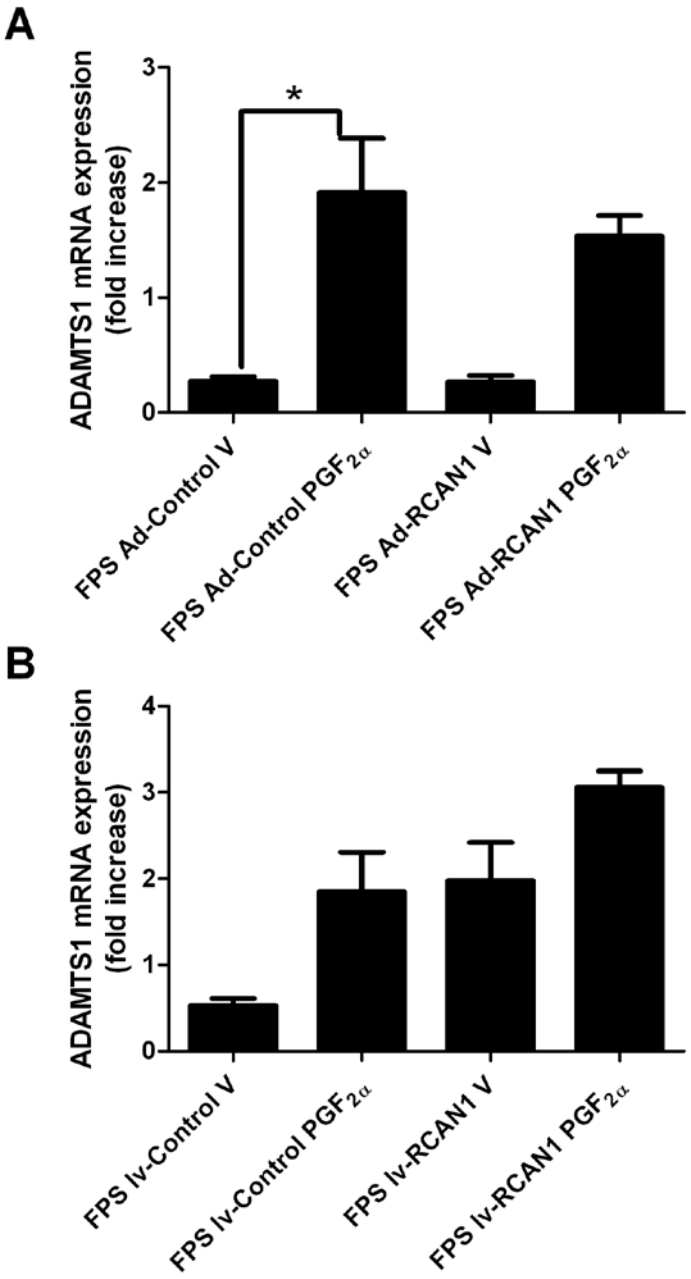


Fig. 34. The role of RCAN1-4 in the regulation of ADAMTS1 expression in FPS cells. FPS Ishikawa cells were transfected with 5MOI of A, adenovirus construct containing non-target (Ad-Control) or RCAN1-4 cDNA (Ad-RCAN1) or B, lentivirus containing non-target control (lv-Control) or RCAN1-4 shRNA (lv-RCAN1). Subsequently, cells were treated with vehicle or $PGF_{2\alpha}$ (100nM) and ADAMTS1 expression was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of 4 independent experiments.

3.4 Discussion

The present study demonstrated elevated expression of ADAMTS1 in poorly differentiated endometrial adenocarcinomas compared with normal endometrium from the proliferative phase of the menstrual cycle. As the endometrium of post-menopausal women is no longer under normal hormonal control, the tissue is atrophic and often not attainable for analysis, we chose normal proliferative phase endometrium as our comparator. This is the phase of the menstrual cycle which exhibits rapid cellular proliferation, differentiation and tissue remodelling and is the phase of the menstrual cycle with the highest expression of FP receptor (Milne and Jabbour, 2003). ADAMTS1 protein was expressed in endometrial vascular cells and glandular epithelial cells. This was similar to the data by Ng et al. who showed that ADAMTS1 expression was localised to the stromal cells surrounding the spiral arteries in the secretory endometrium and glandular epithelial cells (Ng et al., 2006). They provided evidence to suggest that ADAMTS1 is involved in cytokine, IL-1 and TGF, regulation of endometrial decidualisation (Ng et al., 2006). Research by Wen et al. also showed that the expression of ADAMTS1 by endometrial stromal cells is under the control of gonadal steroids DHT and progesterone (Wen et al., 2006). ADAMTS1 has also been shown to be essential for healthy follicle growth which has been attributed to its metalloproteinase properties (Brown et al., 2006). These articles suggest that the metalloproteinase property of ADAMTS1 is involved in endometrial remodelling and together with the data presented in this chapter suggests that ADAMTS1 could be involved in remodelling of the vasculature of endometrial adenocarcinoma and could contribute to its progression.

ADAMTS1 was identified as a target of $PGF_{2\alpha}$-FP signalling in a previous microarray analysis conducted in the Jabbour laboratory. Since ADAMTS1 was expressed in the glandular epithelial and vascular cells of the endometrium, where the FP receptor is expressed, the regulation of ADAMTS1 by $PGF_{2\alpha}$-FP signalling was investigated using an Ishikawa cell line stably transfected with the FP receptor (Fig. 35). ADAMTS1 expression was confirmed to be upregulated by $PGF_{2\alpha}$-FP signalling via a Gq-EGFR-calmodulin-NFAT pathway. In FPS cells, ADAMTS1

expression induced by $PGF_{2\alpha}$ -FP signalling was independent of c-Src, PLC, JNK and ERK1/2. The role of calcium in the regulation of ADAMTS1 is unclear because although the calmodulin inhibitor inhibited ADAMTS1 expression, the calcium and calcineurin inhibitors did not affect ADAMTS1 expression. This may indicate the involvement of co-transcription factor AP-1 which is activated by calmodulin and may regulate NFAT induced gene transcription (Rao et al., 1997).

Recently, Sales et al. found the $PGF_{2\alpha}$-FP signalling upregulates RCAN1-4 expression in FPS cells (Sales, 2009). RCAN1-4 has been shown to negatively regulate NFAT induced CXCL8 expression (Maldonado-Perez et al., 2009). To further elucidate the pathway controlling ADAMTS1 expression in FPS cells, the role of RCAN1-4 in the regulation of NFAT-induced ADAMTS1 in FPS cells was investigated. In FPS cells, RCAN1-4 overexpression had no effect on ADAMTS1 expression and similarly, decreasing RCAN1-4 expression did not affect ADAMTS1 expression. This suggests that ADAMTS1 expression in FPS cells is calcineurin independent because neither RCAN1-4 nor the calcineurin inhibitor CsA affected ADAMTS1 expression induced by $PGF_{2\alpha}$-FP signalling. Therefore, the regulation of ADAMTS1 is likely to involve other intermediary factors. For example, ADAMTS1 gene expression can be controlled by histone deacetylase (HDAC) binding (Chou and Chen, 2008) which can be regulated by calcineurin independent pathways (Martin et al., 2007).

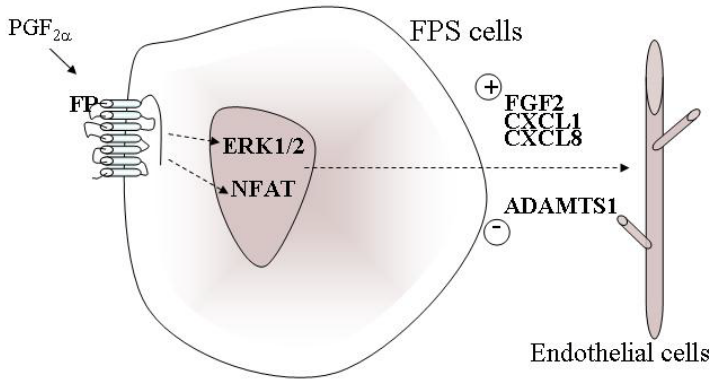


Fig. 35. The regulation of proangiogenic (+) and antiangiogenic (-) factors by $PGF_{2\alpha}$-FP signalling.

In summary, ADAMTS1 mRNA expression is elevated in the mid-late secretory stage of normal endometrium and in poorly differentiated endometrial adenocarcinoma compared with proliferative phase endometrium. ADAMTS1 is prominently expressed in the glandular epithelial and vascular compartments in endometrial adenocarcinoma. In addition, using the endometrial cancer cell line, Ishikawa, stably expressing the FP receptor to levels seen in endometrial cancer (FPS cells), ADAMTS1 expression was found to be regulated by $PGF_{2\alpha}$ signalling in a time dependent manner via a pathway involving the FP receptor, EGFR, calmodulin and NFAT. However, this regulation of ADAMTS1 was not affected by the absence/presence of the NFAT pathway inhibitor, regulator of calcineurin (RCAN1). Data presented in this chapter, together with previous research demonstrating that the metalloproteinase property of ADAMTS1 is involved in endometrial remodelling, indicates that ADAMTS1 could be involved in remodelling of the vasculature during endometrial adenocarcinoma via the mediation of processes such as ECM degradation and cell migration.

4 The role of proangiogenic factors in the regulation of endothelial cell function by $PGF_{2\alpha}$-FP signalling.

4.1 Abstract

The research presented in this chapter demonstrates that conditioned medium, from $PGF_{2\alpha}$ treated Ishikawa cells stably expressing the FP receptor (Ishikawa FPS cells), can increase endothelial cell differentiation (network formation) and proliferation. Treatment of Ishikawa FPS cells with $PGF_{2\alpha}$ increases proangiogenic FGF2, CXCL1 and CXCL8 secretion, which in turn activates FGFR1 and CXCR2 signalling in endothelial cells, and induces the phosphorylation of extracellular signal-regulated kinase (ERK1/2) leading to increased FGF2, CXCL1, CXCL8 and COX-2 expression and secretion of $PGF_{2\alpha}$. Taken together, these data highlight the mechanism by which $PGF_{2\alpha}$–FP receptor signalling can regulate endothelial cell function through the enhancement of an autocrine loop involving FGF2, CXCL1 and CXCL8.

4.2 Introduction

Data generated in our laboratory and others have ascertained a role for the cyclooxygenase (COX)-prostaglandin (PG) axis in the regulation of endometrial adenocarcinomas by increasing cell proliferation and the secretion of angiogenic growth factors (Jabbour et al., 2006b; Tong et al., 2000). This is similar to other cancers where over-expression of COX enzymes and biosynthesis of prostaglandins has been shown to promote cellular proliferation (Tsuji et al., 1996), inhibit apoptosis (Tsujii and DuBois, 1995) and enhance angiogenesis (Tsujii et al., 1998).

Numerous growth factors that enhance angiogenesis have been identified in human endometrial adenocarcinomas. For example, vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factor-2 (FGF2) expression and secretion are elevated in endometrial adenocarcinoma cells (Sales et al., 2007; Sales et al., 2005; Soufla et al., 2008) and both VEGF-A and FGF2 can stimulate angiogenesis in

xenografts in vivo (Danielsen and Rofstad, 1998; Rofstad and Halsor, 2000). However, the molecular mechanisms mediating the role of prostaglandins in regulating vascular function and angiogenesis are still poorly defined. Therefore the aim of this chapter was to investigate the paracrine effects of proangiogenic factors FGF2, CXCL1 and CXCL8, secreted into conditioned medium from $PGF_{2\alpha}$ treated FPS cells (P CM), on endothelial cell functions of differentiation and proliferation.

The data generated in this chapter demonstrates the role of three proangiogenic growth factors FGF2, CXCL1 and CXCL8 in mediating the prostaglandin $F_{2\alpha}$ stimulated effects on endothelial cell functions defined by network formation and proliferation.

4.3 Results

4.3.1 Detection of angiogenic factors in Ishikawa cell conditioned medium

Previous studies using FPS cells have shown that the production of the angiogenic factors vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factor-2 (FGF2) is stimulated by $PGF_{2\alpha}$ treatment but not vehicle treatment and this is not seen in similarly treated WT Ishikawa cells (Sales et al., 2007; Sales et al., 2005). This suggests that the $PGF_{2\alpha}$-FP signalling promotes the production of angiogenic factors. To identify additional angiogenic factors that are upregulated by $PGF_{2\alpha}$ in FPS cells, a commercially available angiogenic array was used. The array membranes were incubated with conditioned medium taken from WT or FPS cells treated with either vehicle or $PGF_{2\alpha.}$ As shown in Fig. 36A there was no difference in secretion of angiogenic proteins between WT cells treated with vehicle (WT V CM) or $PGF_{2\alpha}$ conditioned medium (WT P CM). In contrast, conditioned medium from FPS cells treated with $PGF_{2\alpha}$ (FP P CM) contained observably higher levels of CXCL8, IL6 and CXCL1 when compared with the vehicle treated FPS cell conditioned medium (FP V CM) (Fig. 36B).

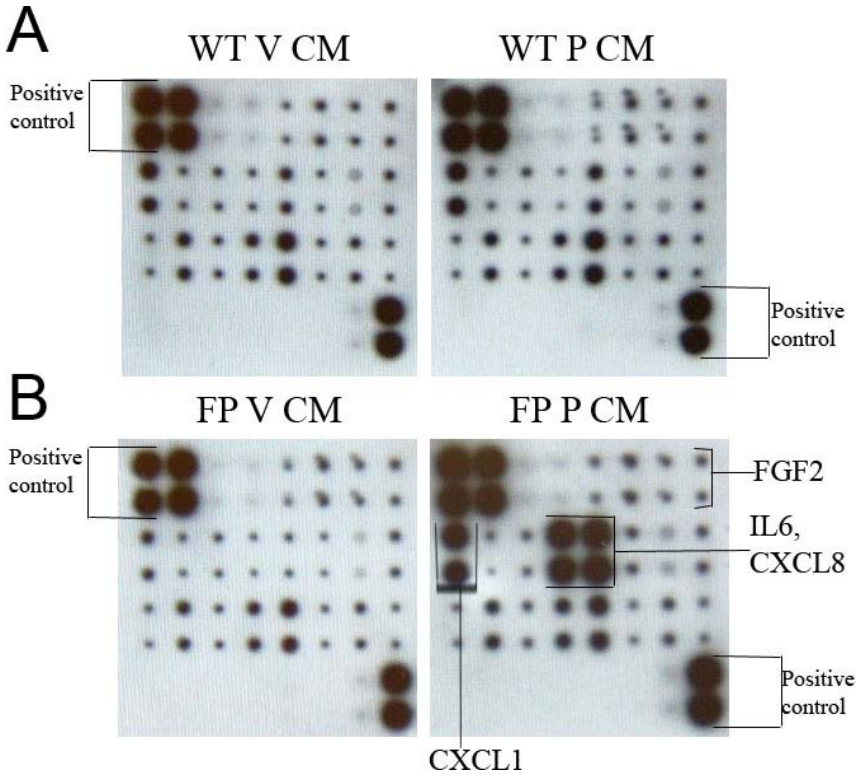


Fig. 36. Angiogenic array of WT and FPS cell conditioned medium. A, Membranes were incubated with vehicle (left; WT V CM) or $PGF_{2\alpha}$ (right; WT P CM) treated conditioned medium from WT Ishikawa cells. B, Membranes were incubated with vehicle (left; FP V CM) or $PGF_{2\alpha}$ (right; FP P CM) treated conditioned medium from FPS Ishikawa cells.

FGF2, CXCL1 and CXCL8 were chosen as the focal angiogenic proteins for this study. The increase in FGF2, CXCL1 and CXCL8 levels in FP P CM compared to FP V CM was confirmed by ELISA (Fig. 37). In order to investigate the contribution of FGF2, CXCL1 and CXCL8 to vascular function, these proteins were immunoneutralised from the FPS cell P CM using specific antibodies and the efficiency of protein immunoneutralisation was confirmed by ELISA (Fig. 37).

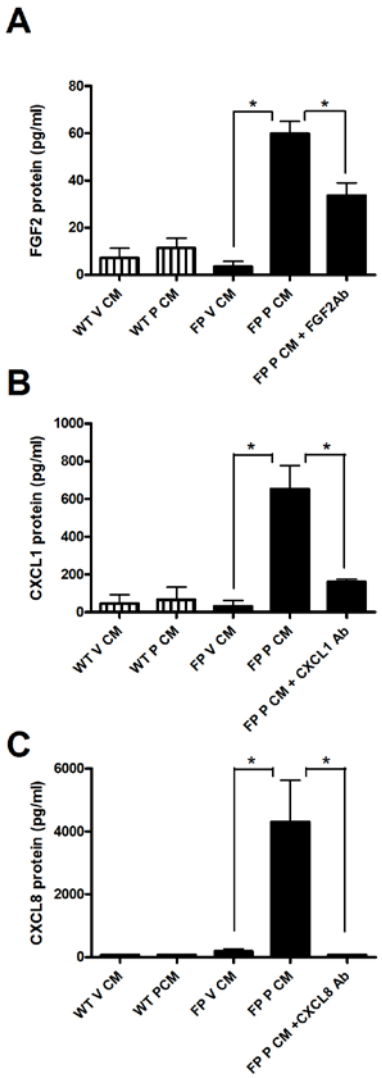


Fig. 37. Angiogenic proteins present in Ishikawa cell conditioned medium. Conditioned medium was collected from WT (striped bars) and FPS (black bars) Ishikawa cells treated with vehicle (V CM) or $PGF_{2\alpha}$ (P CM) for 24hrs. The levels of FGF2 (A), CXCL1 (B) and CXCL8 (C) in the medium were confirmed by ELISA. FGF2, CXCL1 and CXCL8 proteins were immunoneutralised from the FP conditioned medium using specific antibodies (* represents statistical significance $P<0.05$). Data are represented as mean ± SEM from at least three independent experiments.

As shown in Fig. 37, the incubation of FP P CM with respective antibodies, significantly reduced the concentration of FGF2, CXCL1 and CXCL8 in the FP P CM (Fig. 37A, B and C respectively, $P<0.05$).

4.4 The role of FGF2, CXCL1 and CXCL8 in P CM–induced endothelial network formation and proliferation.

For the progression of any tumour larger that approximately 2mm in diameter, a blood supply is needed to provide the tumour cells with enough nutrients and oxygen to meet their metabolic demand (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2000). The sprouting of new blood vessels from existing blood vessels, in order to form a new blood supply, is known as angiogenesis (Hanahan and Folkman, 1996). Endothelial cell network formation and proliferation are two of the processes required for angiogenesis (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2000). As stated by Folkman et al., in vitro models of angiogenesis are useful for identifying the role of individual growth factors or combinations of factors in endothelial network formation and proliferation (Folkman and Haudenschild, 1980). In Fig. 36 and Fig. 37 the angiogenesis array and ELISA, respectively, identified an increased presence of angiogenic FGF2, CXCL1 and CXCL8 in the $PGF_{2\alpha}$ conditioned medium from FPS cells (FP P CM). Since FGF2, CXCL1 and CXCL8 have been shown to regulate vascular function (Li et al., 2003; Presta et al., 2005; Wang et al., 2006), the role of these factors in the FP P CM was examined using endothelial cell network formation and proliferation assays.

Firstly, endothelial cell network formation and proliferation assays were performed using WT and FPS Ishikawa cell conditioned medium treated with vehicle or $PGF_{2\alpha}$ for 24hrs (Fig. 38). The network formation assay was quantified using a method of network branch counting as indicated by the red circle on the photograph in Fig. 38A. Fig. 38A shows a representative photograph of each of the four treatments, WT V CM, WT P CM, FP V CM and FP P CM. The quantification of four independent experiments is displayed in the form of a bar graph below (Fig. 38B). No significant difference in network formation was seen between V CM and P CM from WT Ishikawa cells however the network formation observed with P CM from the FPS cells was significantly increased compared to all other treatments (Fig. 38B, $P<0.05$).

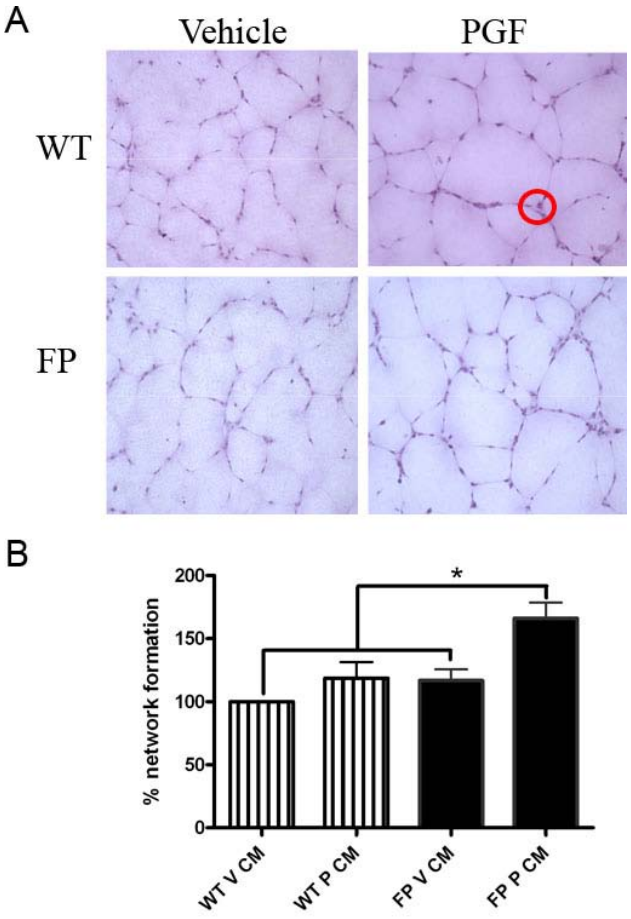


Fig. 38. Endothelial network formation assay with WT and FPS cell conditioned medium. HUVECs, plated on growth factor reduced matrigel, were incubated with WT or FP conditioned medium for 16hrs. The network connections/branches were counted in 10 fields per duplicate A, Images from one representative experiment. Red circle indicates one network branch B, Quantification of branches counted from at least four independent experiments. (* represents statistical significance $P<0.05$). Data are represented as mean ± SEM.

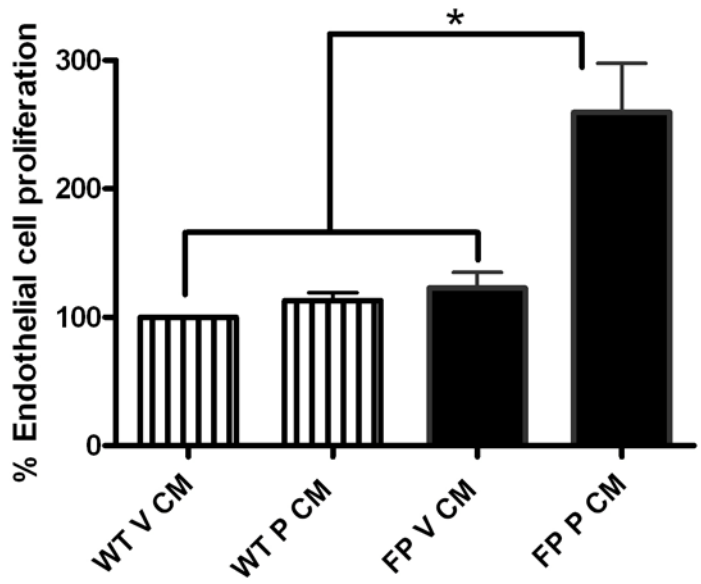


Fig. 39. $PGF_{2\alpha}$-treated FPS cell conditioned medium increases endothelial cell proliferation. HUVECs were treated with vehicle or $PGF_{2\alpha}$ conditioned medium from WT or FPS cells for 96 hours, after which proliferation was assessed using One Solution Reagent (Promega) and absorbance was measured on a spectrophotometer at 490nm. Data from at least four independent experiments. (* represents statistical significance $P<0.05$). Data are represented as mean ± SEM.

Similarly endothelial cell proliferation was not increased by WT P CM compared to WT V CM (Fig. 39). However, FP P CM induced proliferation was significantly increased compared to proliferation in cells treated with FP V CM , WT V CM and WT P CM treatments (Fig. 39, $P<0.05$).

For all further experiments only conditioned medium from the vehicle and $PGF_{2\alpha}$ treated FPS cells (henceforth abbreviated as V CM and P CM respectively) was used as conditioned medium from WT cells did not significantly influence endothelial network formation or proliferation. To identify the role played by FGF2, CXCL1 and CXCL8 in P CM induced endothelial cell function, network formation and proliferation assays were performed using inhibitors of FGF2, CXCL1 and CXCL8 receptors. To confirm the role of FGF2, CXCL1 and CXCL8 in network formation and proliferation, these proteins were immunoneutralised from the CM with their

respective antibodies as shown in Fig. 37. FGF2 signals through FGFR1 and CXCL1 and CXCL8 share the receptor CXCR2. Endothelial cell network formation induced by P CM was significantly reduced by the addition of FGFR1 tyrosine kinase inhibitor, SU4984 and use of FGF2-immunoneutralised P CM (Fig. 40A, $P<0.05$). Notably, SU4984 reduced endothelial network formation to levels below that seen with V CM suggesting it affected basal network formation. Recombinant FGF2 protein (50ng/ml), used as a positive control, increased endothelial network formation to the levels observed with P CM (Fig. 40A, $P<0.05$). Similarly, P CM induced network formation was inhibited by addition of CXCR2 antagonist, SB225002 and CXCL1 or CXCL8 immunoneutralisation from P CM (Fig. 40B, $P<0.05$). Combined immunoneutralisation of CXCL1 and CXCL8 did not have and additive inhibitory effect on P CM induced network formation compared with CXCL1 or CXCL8 alone. Recombinant CXCL1 (10ng/ml) and CXCL8 (10ng/ml) increased endothelial cell network formation compared to control (Fig. 40B, $P<0.05$).

Endothelial cell proliferation induced by P CM was significantly reduced by the addition of SU4984 or use of FGF2-immunoneutralised P CM (Fig. 41A, $P<0.05$). Recombinant FGF2 protein, used as a positive control, also induced endothelial proliferation to a similar level as P CM (Fig. 41A). Similarly, P CM induced proliferation was inhibited, to below basal levels, by addition of SB225002 or CXCL1 or CXCL8 immunoneutralisation from P CM (Fig. 41B, $P<0.05$). Recombinant CXCL1 and CXCL8 were used as positive controls and increased endothelial cell proliferation to levels seen with P CM treatment (Fig. 41B).

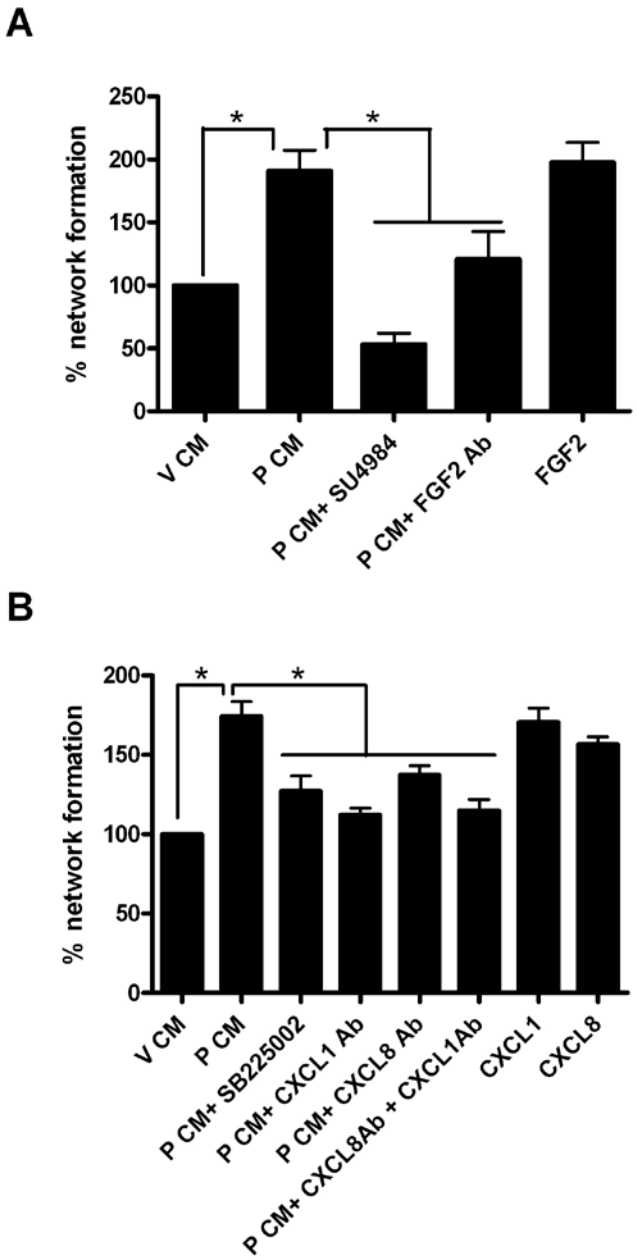


Fig. 40. Network formation using immunoneutralised conditioned medium. Network formation was assessed with A, HUVECs treated with V CM, P CM, P CM and SU4984 (FGFR1 inhibitor) or FGF2-immunoneutralised P CM and B, HUVECs treated with V CM, P CM, P CM and SB225002 (CXCR2 antagonist) or CXCL1/CXCL8-immunoneutralised P CM. Data from at least four independent experiments. (* represents statistical significance $P<0.05$). Data are represented as mean ± SEM.

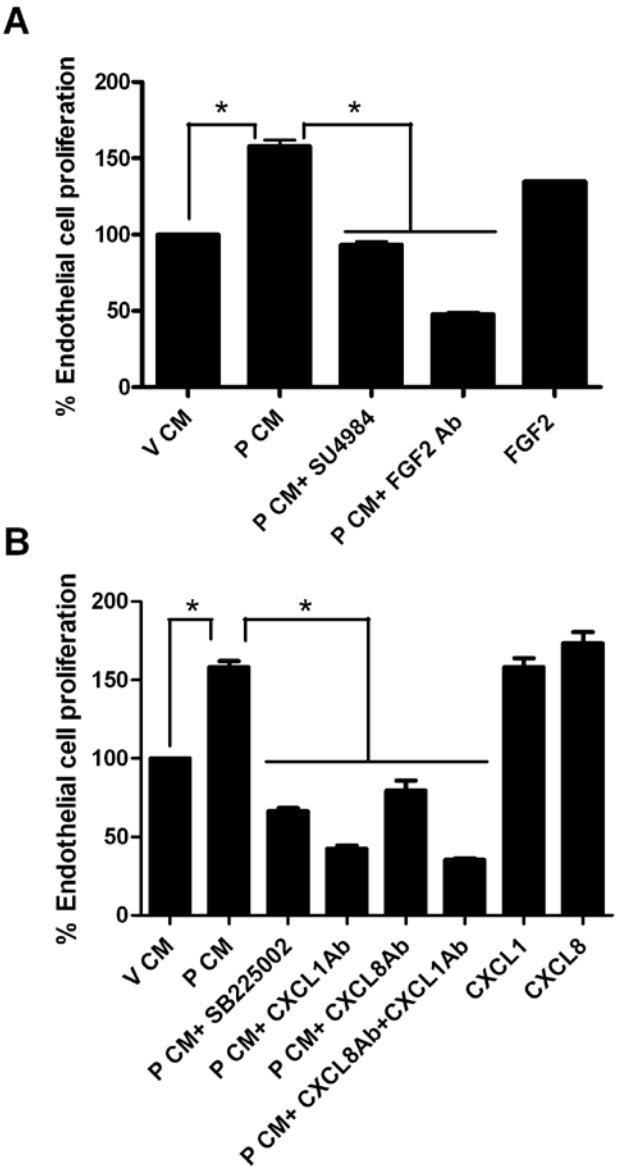


Fig. 41. Proliferation using immunoneutralised conditioned medium. A, HUVECs were treated with V CM, P CM, P CM and SU4984 (FGFR1 inhibitor) or FGF2-immunoneutralised P CM. B, HUVECs were treated with V CM, P CM, P CM and SB225002 (CXCR2 antagonist) or CXCL1/CXCL8-immunoneutralised P CM. After 96hrs proliferation was assessed after the addition of One Solution Reagent (Promega) and absorbance was measure with a spectrophotometer at 490nm. Data from at least three independent experiments represented at mean ± SEM. (* represents statistical significance $P<0.05$).

4.4.1 The effect of P CM on endothelial ERK1/2 phosphorylation

To further investigate the signal transduction pathways involved in endothelial network formation and proliferation initiated after P CM treatment, Western blotting for ERK1/2 phosphorylation was performed. In endothelial cells, increased levels of ERK1/2 phosphorylation have been shown after activation of the FGFR1 and CXCR2 receptors by their respective ligands (MacManus et al., 2007; Presta et al., 2005; Shyamala and Khoja, 1998). To investigate ERK1/2 phosphorylation in response to FPS cell conditioned medium, HUVECs were treated with V CM or P CM for 0, 5, 10, 15, 20 and 30 mins (Fig.42A). Treatment of HUVECs with P CM significantly increased ERK1/2 phosphorylation in a time-dependent manner which was maximal after 10 mins of stimulation, compared to V CM (Fig.42A, $P<0.05$). When FGF2, CXCL1 or CXCL8 were immunoneutralised from the conditioned medium using antibodies specific for each respective protein, there was a significant reduction in ERK1/2 phosphorylation (Fig.42B, $P<0.05$). Goat IgG was used as a control for immunoneutralised P CM and did not interfere with P CM induced ERK1/2 phosphorylation. Recombinant FGF2 was used as a positive control as it is known to phosphorylate ERK1/2 (Sulpice et al., 2002) as shown in Fig.42B. This suggests that FGF2, CXCL1 and CXCL8 all play a role in the activation of ERK1/2 by P CM treatment.

ERK1/2 activation is known to be mediated by upstream adapter proteins and signalling molecules including c-Src, phosphoinositide-3-kinase (PI3K), Akt, protein kinase C (PKC) and raf (Pearson et al., 2001). To further investigate the signalling intermediaries involved in growth factor induced ERK1/2 phosphorylation, HUVECs were treated with P CM in the absence or presence of FGFR1 tyrosine kinase inhibitor (SU4984), CXCR2 antagonist (SB225002), SU4984 and SB225002 combined, ERK1/2 inhibitor (PD98059) or c-Src inhibitor (PP2). All chemical inhibitors, listed above, significantly reduced the P CM-stimulated phosphorylation of ERK1/2 (Fig. 43, $P<0.05$).

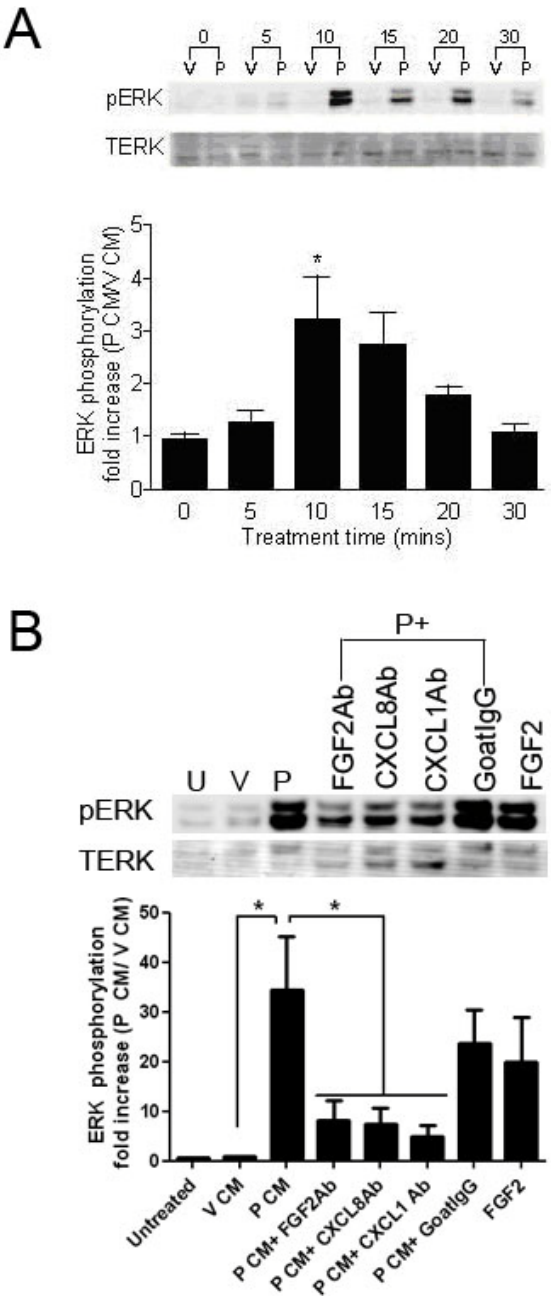


Fig.42. Immunoblots of P CM induced ERK1/2 phosphorylation in HUVECs. A, HUVECs were treated with V CM (V) or P CM (P) for 0,5,10,15,20 and 30 minutes. B, HUVECs were left untreated (U) or treated for 10 min with V CM, P CM, P CM immunoneutralised with FGF2, CXCL8 or CXCL1 antibodies. Cell lysates were subjected to immunoblot analysis using antibodies against phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel). A representative Western blot is displayed with a graph of semi-quantitative analysis of ERK1/2 phosphorylation determined as described in Materials and Methods. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from four independent experiments.

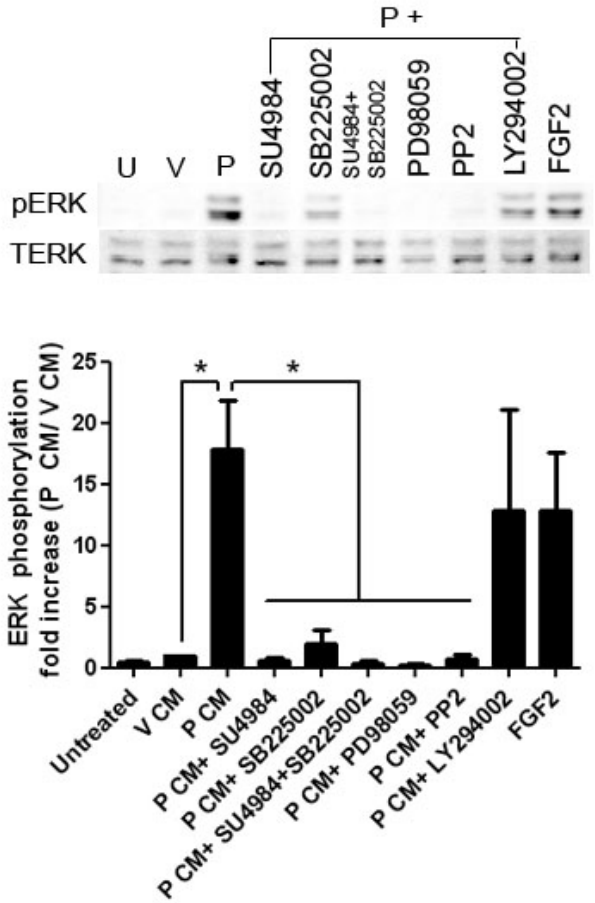


Fig. 43. The effect of conditioned medium on ERK1/2 phosphorylation. HUVECs were left untreated (U) or treated with V CM (V) or P CM (P) for 10 min in the absence/presence of SU4984, SB225002, SU4984+SB225002, PD98059, PP2 or LY294002. Cell lysates were subjected to immunoblot analysis using antibodies against phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel). A representative Western blot is displayed with a graph of semi-quantitative analysis of ERK phosphorylation determined as described in Materials and Methods. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from four independent experiments.

However, treatment of HUVECs with P CM in the presence of the PI3K inhibitor LY294002 did not significantly reduce the phosphorylation of ERK1/2 by P CM (Fig. 43) implying that ERK1/2 activation is independent of PI3K but requires FGFR1, CXCR2 and c-Src activation. Recombinant FGF2 was used as a positive control for ERK1/2 phosphorylation.

4.4.2 The effect of P CM on angiogenic gene expression.

ERK1/2 is a potent regulator of cell proliferation and can increase the transcription of genes promoting cell growth in many cell types (Pearson et al., 2001). In order to investigate if P CM treatment induced an upregulation in angiogenic gene expression via ERK1/2, which could enhance endothelial cell function in an autocrine manner, HUVECs were treated with V CM or P CM for 1,2,3,4,6,16 and 24 hrs. A significant increase in the levels of FGF2, CXCL1 and CXCL8 mRNA expression was found after 3hrs of treatment with P CM compared to V CM treatment (Fig. 44 A, B and C respectively, $P<0.05$).

To investigate whether the angiogenic factors FGF2, CXCL1 and CXCL8 autoregulate their own expression in a positive feedback mechanism, HUVECs were treated with P CM and small molecule chemical inhibitors against the FGFR1 receptor (SU4984) and CXCR2 receptor (SB225002) and ERK1/2 (PD98059). FGF2, CXCL1 and CXCL8 mRNA expression was significantly decreased when HUVECs were treated with P CM in the presence of inhibitors SU4984, SB225002 and PD98059, compared to P CM treatment alone (Fig. 45 A, B and C respectively, $P<0.05$). This suggests that the transcription of these angiogenic factors requires the activation of FGFR1 and CXCR2, by their respective ligands, as well as activation of ERK1/2.

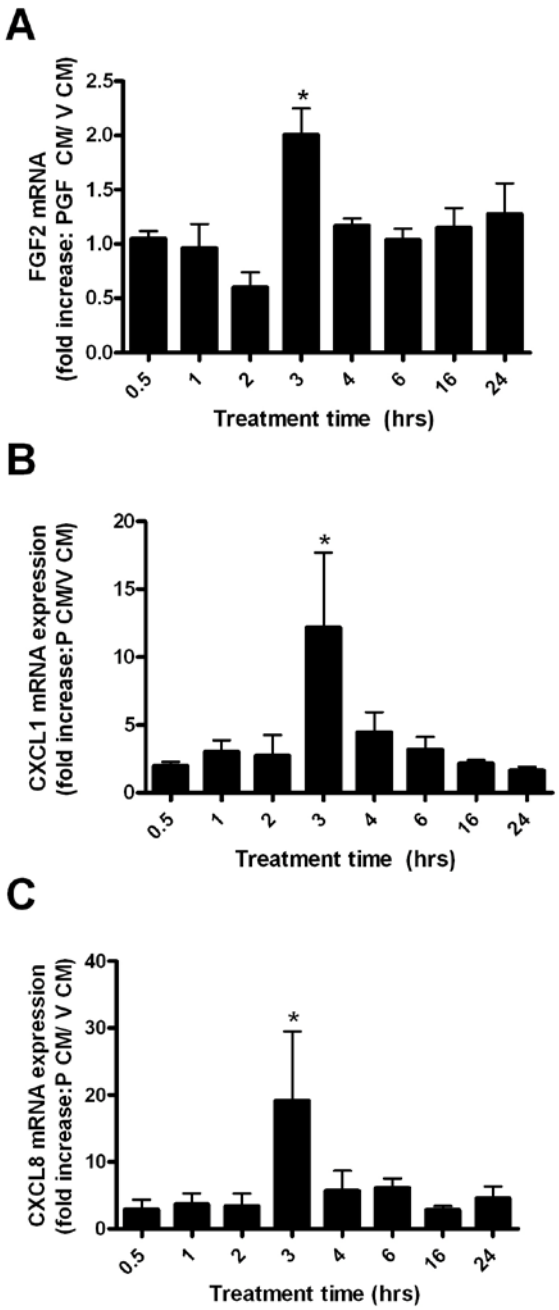


Fig. 44. P CM treatment upregulates angiogenic genes in HUVECs. HUVECs were treated with V CM or P CM for 0.5,1,2,3,4,6,16,24 hrs and mRNA expression of FGF2 (A), CXCL1 (B) and CXCL8 (C) was determined by RT-PCR. (* represents statistical significance from V CM; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

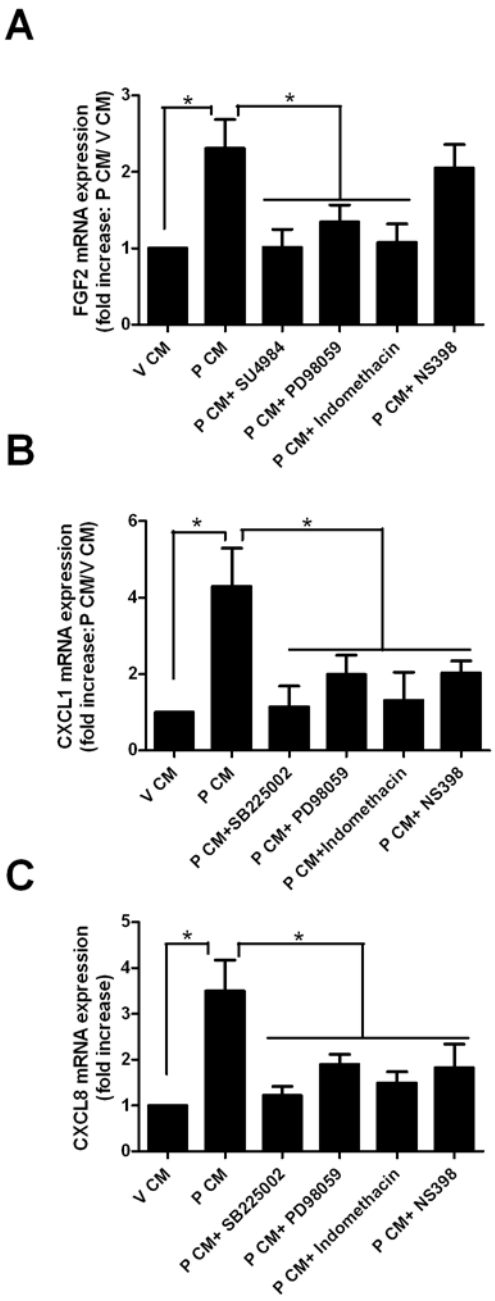


Fig. 45. Angiogenic factor mRNA expression is dependent on receptor signalling, cyclooxygenases and ERK1/2. HUVECs were treated for 3hrs with V CM or P CM in the absence/presence of SU4984 (FGFR1 tyrosine kinase inhibitor), PD98059 (ERK1/2 inhibitor), Indomethacin (COX inhibitor) or NS398 (COX-2 inhibitor) and mRNA expression of FGF2 (A), CXCL1 (B) and CXCL8 (C) was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

Previous research has indicated the cyclooxygenase (COX) enzymes (Dubois et al., 1998) can be involved in an additional step in the upregulation of angiogenic factors in endothelial cells and during angiogenesis in vivo (Leahy et al., 2002) (Hernandez et al., 2001) (Majima et al., 2000). For example, Leahy et al. found that VEGF-A expression was reduced by COX-2 inhibitor NS389 in endothelial cells. Research by Majima et al., using the sponge angiogenesis rat model, indicates that FGF2-induced angiogenesis is augmented by an increase in VEGF-A production which can be inhibited by the specific COX-2 inhibitor NS398 (Majima et al., 2000). It is generally understood that COX-1 is the constitutively expressed enzyme regulating cellular homeostatic prostaglandin production whereas, COX-2 is the inducible enzyme upregulated by growth factor stimulation (Dubois et al., 1998; Masferrer et al., 2000), however both COX-1 and COX-2 have been implicated in tumour angiogenesis (Sales et al., 2002; Tsujii et al., 1998). To investigate the possibility that COX-1 and COX-2 may be involved in the upregulation of angiogenic genes, a general COX inhibitor Indomethacin and a specific COX-2 inhibitor, NS398, were used. HUVECs were co-treated for 3hrs with P CM and Indomethacin or NS398 after which FGF2, CXCL1 and CXCL8 mRNA expression was examined. Interestingly, the addition of Indomethacin a general cyclooxygenase (COX) inhibitor to P CM decreased FGF2, CXCL1 and CXCL8 mRNA expression suggesting a role for both cyclooxygenases in growth factor production (Fig. 45 A, B and C respectively, $P<0.05$). The use of the specific COX-2 inhibitor (NS398) decreased CXCL1 and CXCL8 gene expression (Fig. 45 A, B and C, $P<0.05$) but not FGF2 expression.

As the COX inhibitors prevented FGF2, CXCL1 and CXCL8 mRNA expression, the mRNA expression levels of COX-1 and COX-2 in HUVECs treated with V CM or P CM were compared. COX-1 mRNA levels were not elevated at any of the time points tested (Fig. 46A). In contrast COX-2 mRNA levels were elevated after 3hrs of treatment with P CM compared to V CM treatment (Fig. 46B). As with the angiogenic genes FGF2, CXCL1 and CXCL8, COX-2 mRNA expression was inhibited by the addition of inhibitors of FGFR1, CXCR2 and ERK1/2 suggesting

that the upregulation of COX-2 is induced by growth factors in the P CM, which then activate ERK1/2 via their respective receptors (Fig.47).

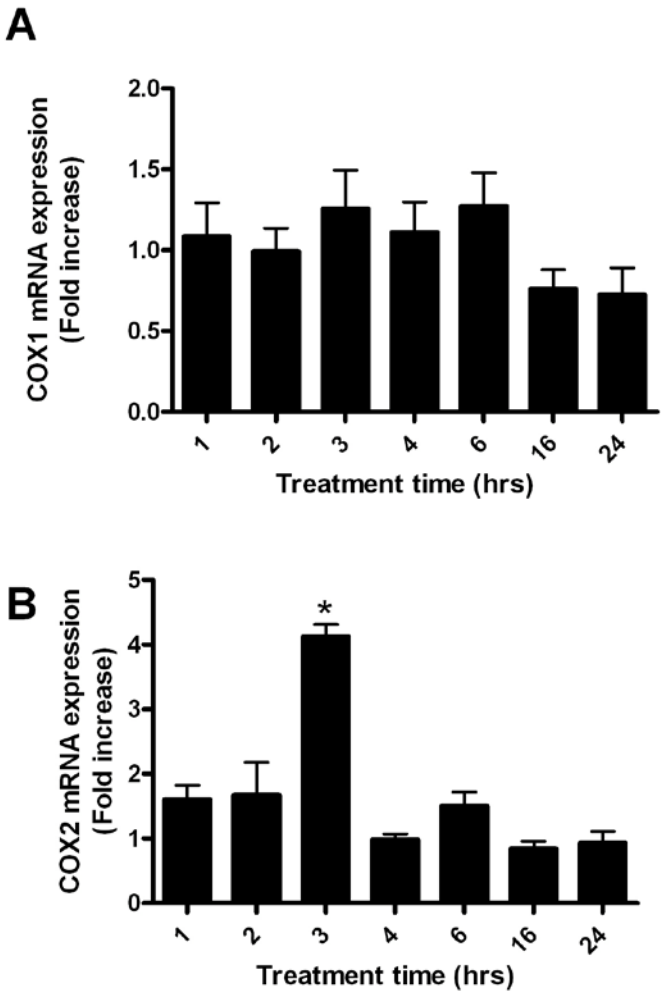


Fig. 46. The effect of P CM treatment on COX-1 and COX-2 expression. HUVECs were treated with V CM or P CM for 1,2,3,4,6,16,24 hrs and mRNA expression of COX-1 (A) and COX-2 (B) was determined by RT-PCR. (* represents statistical significance from V CM; $P<0.05$). Data are represented as mean ± SEM (fold increase V CM/ P CM) from 5 independent experiments.

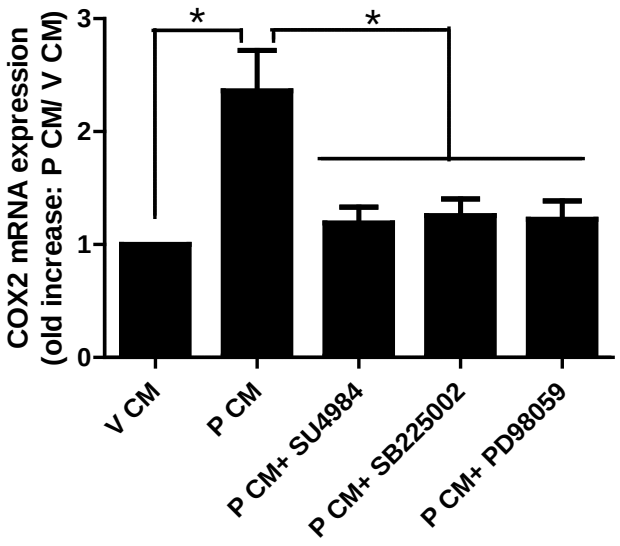


Fig.47. P CM growth factor signalling mediates COX-2 expression. HUVECs were treated for 3hrs with V CM or P CM in the absence/presence of SU4984 (FGFR1 tyrosine kinase inhibitor), SB225002 (CXCR2 antagonist) or PD98059 (ERK1/2 inhibitor) and mRNA expression of COX-2 was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

4.4.3 ERK1/2 and cyclooxygenases (COX) are involved in P CM induced endothelial cell function.

The previous data demonstrate that ERK1/2, COX-1 and COX-2 are involved in the increased expression of angiogenic factors as a result of FGF2, CXCL1 and CXCL8 signalling in endothelial cells. Following on from this, the functional relevance of ERK1/2 and COX-1 and COX-2 in P CM-induced endothelial cell network formation and proliferation was investigated. Specific inhibitors for both COX-1 (SC560) and COX-2 (NS398) were used in order to clearly identify any differential effects these two enzymes may have on endothelial cell network formation and proliferation. HUVECs were treated with V CM or P CM in the presence or absence of PD98059 (ERK1/2 inhibitor), SC560 and NS398. The ERK1/2 inhibitor and both COX inhibitors significantly reduced the P CM-mediated increase in network formation (Fig. 48A, $P<0.05$). Similarly, co-treatment with P CM and PD98059,

SC560 or NS398 significantly decreased the P CM-mediated endothelial cell proliferation (Fig. 48B, $P<0.05$).

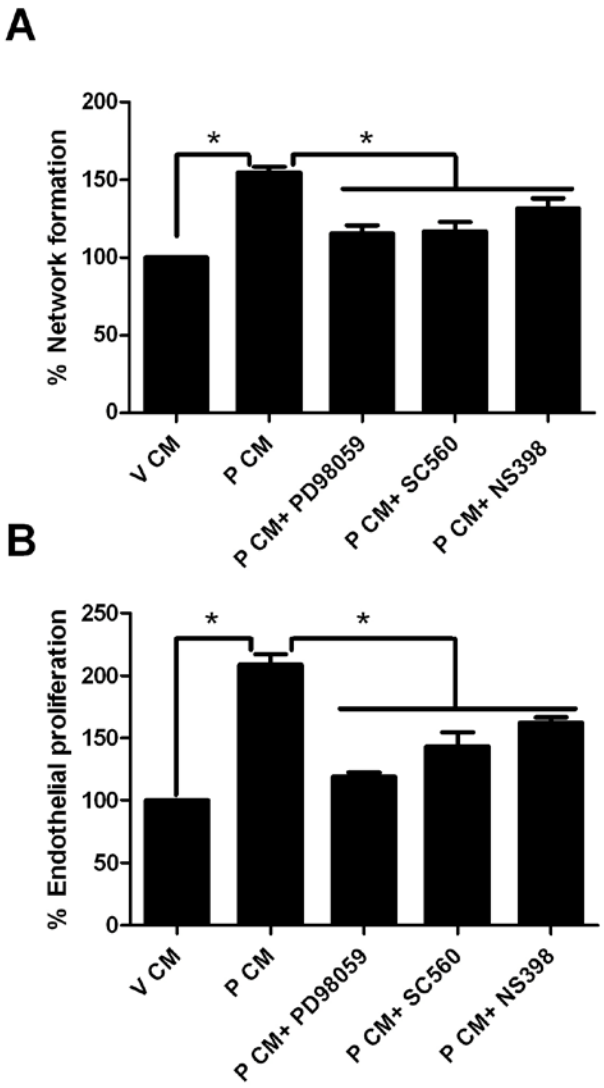


Fig. 48. ERK1/2, COX-1 and COX-2 are involved in endothelial cell function. A, HUVECs were seeded on matrigel containing PD98059 (MEK inhibitor), SC560 (COX-1 inhibitor) or NS398 (COX-2 inhibitor) and the network assay was performed. B, HUVECs were treated with V CM and P CM plus or minus PD98059, SC560 or NS398 for 96hrs afterwhich proliferation was assessed by measuring absorbance (490nm) following the addition of One Solution Proliferation Reagent (Promega). (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from four independent experiments.

4.4.4 Endothelial secretion of prostaglandins induced by CM treatment.

The cyclooxygenase enzymes catalyse the rate limiting step in the synthesis of prostaglandins (PG) (Dubois et al., 1998). Prostaglandins are secreted by endothelial cells and in vitro data along with in vivo data suggests that they can promote angiogenesis by enhancing endothelial cell proliferation, migration and network formation (Churchman et al., 2007; Finetti et al., 2008; Jabbour et al., 2006b; Majima et al., 2003). Since, COX-2 was upregulated by P CM, the secretion of PGE_2 and $PGF_{2\alpha}$ was examined. HUVECs were treated with V CM or P CM for 0, 3, 6, 16, 24, 48 and 72 hrs and the secretion of PGE_2 (Fig. 49A) and $PGF_{2\alpha}$ (Fig. 49B) was measured by ELISA. There was no significant difference in the levels of endothelial PGE_2 secreted by HUVECs in response to CM at any time point tested (Fig. 49A). In contrast, the amount of endothelial $PGF_{2\alpha}$ was elevated in HUVECs at 6hrs following P CM treatment compared to both V CM treatment (6hrs) and P CM at time 0hrs (Fig. 49B, $P<0.05$).

To confirm the involvement of COX enzymes in the secretion of $PGF_{2\alpha}$, HUVECs we treated for 6hrs with P CM in the presence of the general COX inhibitor Indomethacin, a specific COX1 inhibitor (SC560) or a specific COX-2 inhibitor (NS398) (Fig. 50). We found that the P CM-induced secretion of $PGF_{2\alpha}$ was significantly reduced by co-treatment of cells with NS398 or Indomethacin (Fig. 50, $P<0.05$). Interestingly, despite there being no increase in COX-1 mRNA expression, the specific COX-1 inhibitor SC560 inhibited endothelial cell prostaglandin $F_{2\alpha}$ secretion (Fig. 50, $P<0.05$) implying COX-1 may be necessary for basal levels of prostaglandin production.

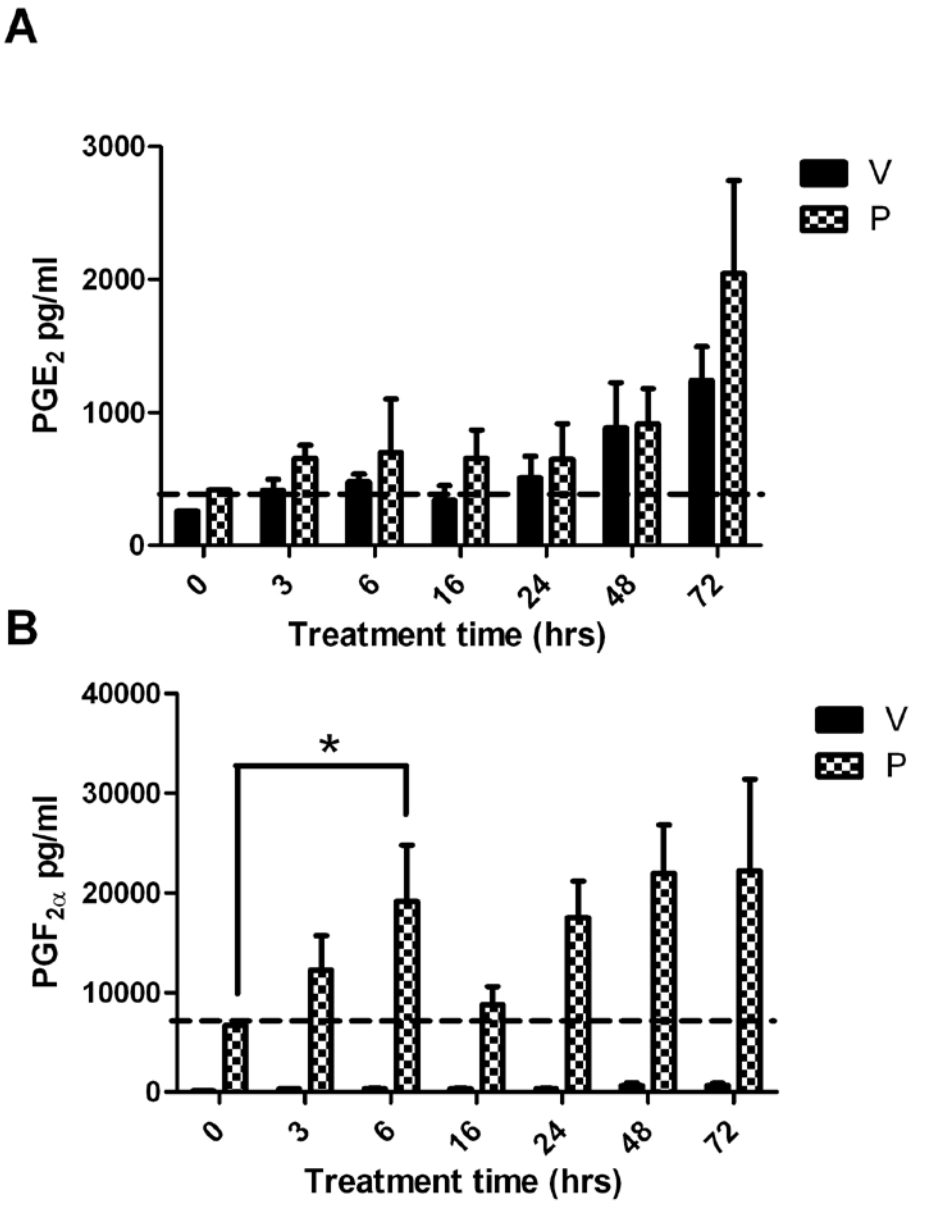


Fig. 49. Endothelial secretion of prostaglandins stimulated by P CM. HUVECs were incubated with V CM or P CM for 0, 3, 6, 16, 24, 48 and 72 hrs and thereafter conditioned medium from HUVECs was collected and used for PGE_2 (A) and $PGF_{2\alpha}$ (B) ELISA analysis as described in the methods. (* represents statistical significance $P<0.05$) Data are represented as mean ± SEM from at least three independent experiments.

Fig.47 showed that COX-2 mRNA expression was elevated by P CM via a mechanism involving the activation of FGFR1, CXCR2 and ERK1/2. As COX-2 is

the inducible enzyme involved in prostaglandin production, the effect of inhibitors of FGFR1 (SU4984), CXCR2 (SB225002) and ERK1/2 (PD98059) on P CM-induced endothelial $PGF_{2\alpha}$ secretion was examined. The addition of SU4984 (FGFR1 inhibitor), SB225002 (CXCR2 antagonist) and PD98059 (MEK inhibitor) to P CM significantly reduced the increase in endothelial $PGF_{2\alpha}$ secretion seen with P CM treatment alone (Fig. 50, $P<0.05$). This suggests that FGF2, CXCL1 and CXCL8 signal via their receptors to ERK1/2 and COX-2 thereby increasing $PGF_{2\alpha}$ secretion.

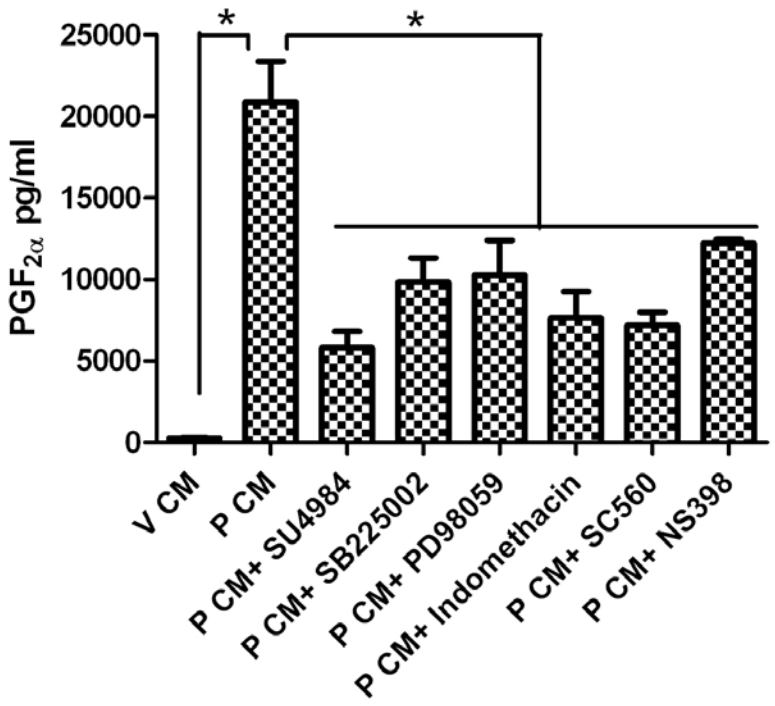


Fig. 50. Secretion of endothelial $PGF_{2\alpha}$ inhibited by P CM signalling. HUVECs were treated with V CM or P CM with or without SU4984 (FGFR1 inhibitor), SB225002 (CXCR2 antagonist), PD98059 (ERK1/2 inhibitor), Indomethacin (COX inhibitor), SC560 (COX-1 inhibitor) and NS398 (COX-2 inhibitor) for 6hrs after which the concentration of $PGF_{2\alpha}$ in the HUVEC medium was determined by ELISA. (* represents statistical significance $P<0.05$) Data are represented as mean ± SEM from three independent experiments.

4.4.5 Discussion

During tumour angiogenesis, the sprouting of new blood vessels from existing blood vessels is augmented by the ability of endothelial cells to proliferate, migrate and differentiate (Adams and Alitalo, 2007; Ausprunk and Folkman, 1977; Folkman and Haudenschild, 1980; Hanahan and Folkman, 1996). Factors secreted by the tumour cells that enhance these endothelial cell functions required for effective blood vessel formation, will enable the tumour to better meet its metabolic demand thereby enhancing tumour progression (Adams and Alitalo, 2007; Hanahan and Weinberg, 2000). The endothelial cell network formation and proliferation assays have been extensively used as in vitro models to investigate factors influencing angiogenesis (Folkman and Haudenschild, 1980; Goodwin, 2007; Tsujii et al., 1998).

Data in this chapter demonstrate that conditioned medium from endometrial adenocarcinoma cells, which stably express the FP receptor to the levels observed in endometrial adenocarcinomas (FPS cells) secrete elevated levels of the angiogenic factors FGF2, CXCL1 and CXCL8. Cellular manipulation of receptor expression allows individual signalling pathways to be examined (Abramovitz et al., 2000). Hence, the overexpression of the FP receptor facilitates the study of the $PGF_{2\alpha}$-FP receptor signalling pathway. Data herein shows that the angiogenic factors, FGF2, CXCL1 and CXCL8, promote endothelial network formation (differentiation) and proliferation. Previous in vitro studies have shown that FGF2, CXCL1 and CXCL8 can all influence endothelial cell function (Kanda et al., 1997; Li et al., 2003; Li et al., 2005; Wang et al., 2006). For example, Kanda et al. found that recombinant FGF-2 treatment of HUVECs enhances endothelial cell network formation and proliferation and Li et al. showed that treatment of HUVECs with recombinant CXCL8 enhances endothelial cell network formation and proliferation (Li et al., 2003). Similarly, Wang et al. showed that recombinant CXCL1 induced bovine lung microvascular endothelial cell migration and CXCL1 immunoneutralisation from colon cancer cell conditioned medium inhibited endothelial cell network formation.

In vivo and ex vivo data shows that, FGF2, CXCL1 and CXCL8 are all expressed by epithelial and endothelial cells of the human endometrium (Luk et al., 2005; Mulayim et al., 2003; Sales et al., 2007; Wallace et al., 2009). As demonstrated in this chapter using a specific FGFR1 tyrosine kinase inhibitor, CXCR2 antagonist and FGF2-, CXCL1- and CXCL8-immunoneutralised conditioned medium, the effects of conditioned medium on endothelial cell network formation and proliferation could be mediated via FGF2-FGFR1 signalling as well as CXCL1/CXCL8-CXCR2 signalling. The immunoneutralisation of each of these angiogenic factors inhibited endothelial cell function and is in agreement with in vivo data from Rofstad et al., who showed that immunoneutralisation of VEGF-A, FGF2 or IL-8 from melanoma cell xenografts significantly reduced angiogenesis (Rofstad and Halsor, 2000). There was no cross reactivity between the CXCL1 and CXCL8 antibodies as CXCL8 was not immunoneutralised by the CXCL1 antibody and vice versa. In another study by Giavazzi et al. using an endometrial cancer xenograft model, FGF2-induced tumour growth and angiogenesis required the expression of VEGF-A (Giavazzi et al., 2003), demonstrating that the coordination of growth factors is needed for angiogenesis. These data indicate that proangiogenic growth factors synergise to promote angiogenesis which is either reinforced by an additive effect of these factors or protected by a mechanism of redundancy to ensure the survival of the tumour.

ERK1/2 signalling is known to be a potent regulator of cell growth, differentiation and development (Lewis et al., 1998). ERK1/2 is modulator of gene transcription activated by phosphorylation cascades which can be induced by most growth factors (Pearson et al., 2001; Turjanski et al., 2007). FGF2, CXCL1 and CXCL8 have all been shown to induce ERK1/2 phosphorylation in a variety of cell types (MacManus et al., 2007; Shyamala and Khoja, 1998; Sulpice et al., 2002). This chapter investigated the conditioned medium signalling to ERK1/2 in HUVECs and found within this experimental paradigm that ERK1/2 was phosphorylated in a time dependent manner via the FGFR1 signalling to c-Src, independent of PI3K. This is in agreement with the observations of Sulpice et al. who showed that, in adrenal cortex capillary endothelial cells, ERK1/2 phosphorylation induced by recombinant

FGF2 is not mediated via the PI3K pathway (Sulpice et al., 2002). c-Src is a protein tyrosine kinase which co-ordinates a diverse spectrum of receptor-induced signalling to ERK1/2 via the phosphorylation of signalling intermediates such as Ras and Raf (Thomas and Brugge, 1997). c-Src has been shown to be involved in FGF2-induced angiogenesis (Kilarski et al., 2003) and a recent study has shown that c-Src, Raf and ERK1/2 are essential for HUVEC lumen formation in vitro (Koh et al., 2009). ERK1/2 phosphorylation was inhibited by CXCL1 or CXCL8 immunoneutralisation and the CXCR2 antagonist suggesting that CXCL1/CXCL8 signalling to CXCR2 promotes ERK1/2 activation. This ERK1/2 activation via CXCL1 and CXCL8 may also involve c-Src as CXCR2 has been shown to co-localise with and transactivate VEGF-A receptor tyrosine kinase, VEGFR2 in a c-Src dependent mechanism (Petreaca et al., 2007). If CXCR2 can transactivate VEGFR2 and EGFR it is possible it may act similarly with FGFR1. This could be an interesting focus for further investigations.

Once phosphorylated by growth factors, activated ERK1/2 can translocate to the nucleus and promote the transcription of additional growth factors (Turjanski et al., 2007). For example, ERK1/2 was shown to phosphorylate the transcription factor SP1 at two threonine sites leading to increased expression of vascular endothelial growth factor (Milanini-Mongiat et al., 2002). As ERK1/2 was found to be activated by FGF2, CXCL1 and CXCL8 in the P CM, the mRNA expression of endothelial FGF2, CXCL1 and CXCL8 was examined after P CM treatment. All three factors were upregulated after three hours of P CM treatment. The use of inhibitors of ERK1/2 and growth factor receptors, FGFR1 and CXCR2, confirmed that FGF2 expression was increased by P CM treatment via FGF2-FGFR1 activating ERK1/2. This indicates that ERK1/2 is involved in a positive signalling loop promoting FGF2 expression. In support of this, FGF2 was shown to be upregulated in epithelial cells via ERK1/2 activation after treatment with recombinant FGF2 as part of an autocrine loop (Sales et al., 2007). Similarly, in this chapter CXCL1 and CXCL8 expression was also upregulated by CXCL1/CXCL8 signalling via CXCR2 and activating ERK1/2. Previous studies have shown that, CXCL1 and CXCL8 mRNA is

upregulated in an ERK1/2 dependent manner after cytokine treatment (Matsumiya et al., 2002; Yang et al., 2008). For example, in HeLa cells treated with IL-1β, the upregulation of CXCL1 and CXCL8 expression is decreased by ERK1/2 inhibition (Yang et al., 2008). In addition, Matsumiya et al. showed that HUVEC expression of CXCL1 is stimulated by treatment with a soluble IL-6 receptor in an ERK1/2 dependent manner (Matsumiya et al., 2002). The upregulation of FGF2, CXCL1 and CXCL8 was transient implying that their activation is tightly regulated. This could be via the regulation of ERK1/2 by phosphatases such as hematopoietic protein-tyrosine phosphatase (HePTP), mitogen-activated protein kinase phosphatase-3 (MKP3), and protein serine/threonine phosphatase 2A (PP2A) which can all dephosphorylate and hence deactivate ERK1/2 (Turjanski et al., 2007). For example, in a previous report, phosphorylation of ERK1/2 by Epidermal Growth Factor and FGF2 stimulation was reversed by PP2A/Bα and Bδ dephosphorylation of ERK1/2 (Van Kanegan et al., 2005).

The cyclooxygenases are enzymes responsible for production of prostaglandins and have been shown to promote growth factor induced angiogenesis in vitro and in vivo (Amano et al., 2002; Leahy et al., 2002; Tsujii et al., 1998). Endothelial expression of FGF2, CXCL1 and CXCL8 was inhibited by addition of a general COX inhibitor. In addition, CXCL1 and CXCL8 expression was inhibited by a specific COX-2 inhibitor suggesting that COX-1 and COX-2 may both play a role in P CM induced endothelial cell function. The mRNA expression of COX-2, but not COX-1, was upregulated by P CM in an FGFR1, CXCR2 and ERK1/2 dependent manner. This is in agreement with data showing that treatment of microvascular endothelial cells with FGF2 upregulates many angiogenic genes including COX-2 whereas no change in COX-1 expression was observed (Yue et al., 2006). In this thesis, data suggest that COX-2 is being induced by FGF2, CXCL1 and CXCL8 in the P CM via ERK1/2 and that this upregulated COX-2 is inducing CXCL1 and CXCL8 production but not FGF2. It is likely that FGF2 expression is primarily upregulated by ERK1/2 modulation of its gene transcription in an autocrine loop as has been reported for the role of FGF2 in endometrial epithelial cells (Sales et al., 2007). As COX-1 controls

the basal levels of prostaglandin production (Dubois et al., 1998), it is possible that the COX-1 inhibitor is acting to inhibit basal levels of COX-1 production which control basal prostaglandin levels. The basal levels of COX-1 may directly regulate the expression of angiogenic proteins as cytoplasmic cyclooxygenases can induce PPAR-dependent transcription (Bogatcheva et al., 2005).

To confirm the functional relevance of ERK1/2 in endothelial cells, network formation and proliferation assays were performed. With the use of chemical inhibitors, endothelial network formation and proliferation was found to involve the activation of ERK1/2. This confirms the premise that the growth factors FGF2, CXCL1 and CXCL8, acting via their receptors on endothelial cells, can activate the pathway involving c-Src-ERK1/2 leading to upregulation of endothelial growth factors which can facilitate endothelial cell network formation and proliferation in an autocrine positive feedback loop (Fig. 51). Interestingly, FGF2 induced murine brain capillary endothelial cell differentiation was found by Klint et al., to be dependent on c-Src but independent of ERK1/2 (Klint et al., 1999). This could support the accepted notion that growth factor signalling in endothelial cells is tissue type specific or could be a result of the fact that this was an observation and they omitted to quantify their endothelial cell differentiation assay by network branch counting, as they did in their later studies (Klint et al., 1999).

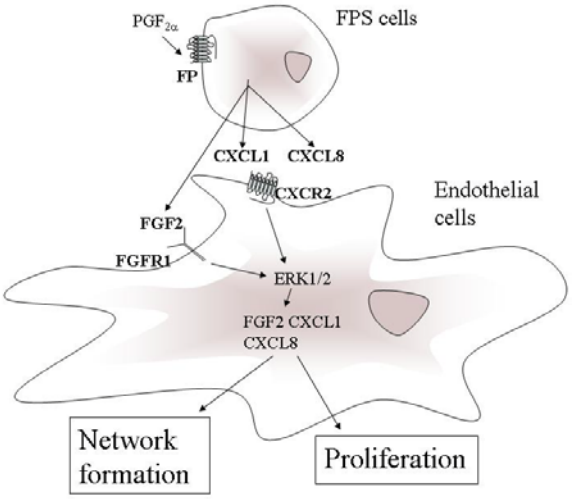


Fig. 51. A schematic representation of P CM induced FGF2, CXCL1 and CXCL8 promoting endothelial cell network formation and proliferation.

Using a general COX inhibitor and a specific COX-2 chemical inhibitor, COX-1 and COX-2 were also found to be involved in P CM-induced endothelial cell network formation and proliferation. Endothelial cyclooxygenase enzymes have previously been shown to be involved in endothelial cell network formation and proliferation (Hernandez et al., 2001; Kentaro Yazawa et al., 2005). For example, the COX-2 inhibitor JTE-522 inhibited HUVEC network formation and proliferation (Kentaro Yazawa et al., 2005). Additionally, Hernandez et al. showed that COX-2 expression induced by VEGF-A treatment in HUVECs was required for endothelial cell network formation (Hernandez et al., 2001). Therefore, in an additional level of complexity, the cyclooxygenases may further increase the expression of FGF2, CXCL1 and CXCL8 facilitating endothelial cell function. The P CM induced elevation of COX-2 expression leads to increased levels of prostaglandins. It is possible that COX-1 is involved in the regulation of basal levels of endothelial network formation and proliferation as COX-1 is generally thought to maintain homeostasis through the production of prostaglandins whereas COX-2 controls the inducible endothelial function (Dubois et al., 1998; Masferrer et al., 2000). Alternatively, a previous report has suggested that the COX-1 inhibitor may not be as specific to COX-1 as first thought because SC560 was found by Brenneis et al. to inhibit TNFα induced PGE_2 production in COX-1 deficient cells (Brenneis et al., 2006). However, in vitro experiments by Tsuji et al. showed that colon cancer cell-stimulated HUVEC network formation involved endothelial COX-1 but not COX-2 (Tsujii et al., 1998). In contrast, in vivo xenograft tumour growth was inhibited by general COX and specific COX-2 inhibitors however, they omitted analysis of the effect of these inhibitors on angiogenesis (Tsujii et al., 1998). Additional in vivo experiments suggest that it is COX-2 that is responsible for inducing angiogenesis for example, an analysis by Leahy et al. (2002) found that FGF2 increased COX-2 protein expression by an estimated 2-3 fold when analysed in an in vivo model (Leahy et al., 2002) and FGF2 driven angiogenesis in the rat cornea was COX-2 but not COX-1 dependent (Masferrer et al., 2000). These findings together with those in this thesis suggest that the prostaglandins produced by both COX-1 and COX-2 may have a role in P CM-induced endothelial cell function.

Prostaglandins have been shown to be secreted by endothelial cells and directly influence endothelial cell function via their receptors on endothelial cells (Finetti et al., 2008; Rao et al., 2007; Tamura et al., 2006). Cytokine stimulation of HUVECs has been shown to increase the secretion of 16keto-$PGF_{1\alpha}$ and PGE_2 in a COX-2 dependent manner (Parfenova et al., 2001), however in this study no elevation in PGE_2 biosynthesis in response to P CM was observed. Instead, endothelial cells were found to secrete elevated levels of $PGF_{2\alpha}$ following activation by CM from $PGF_{2\alpha}$-treated Ishikawa FPS cells and that this $PGF_{2\alpha}$ secretion was regulated via the FGF2-FGFR1- and CXCL1/CXCL8-CXCR2- ERK1/2–mediated induction of COX-2 (see Fig. 52 for a schematic representation). This finding indicates that prostaglandin $F_{2\alpha}$ may be directly involved in endothelial cell network formation and proliferation and this will be explored in chapter 5.

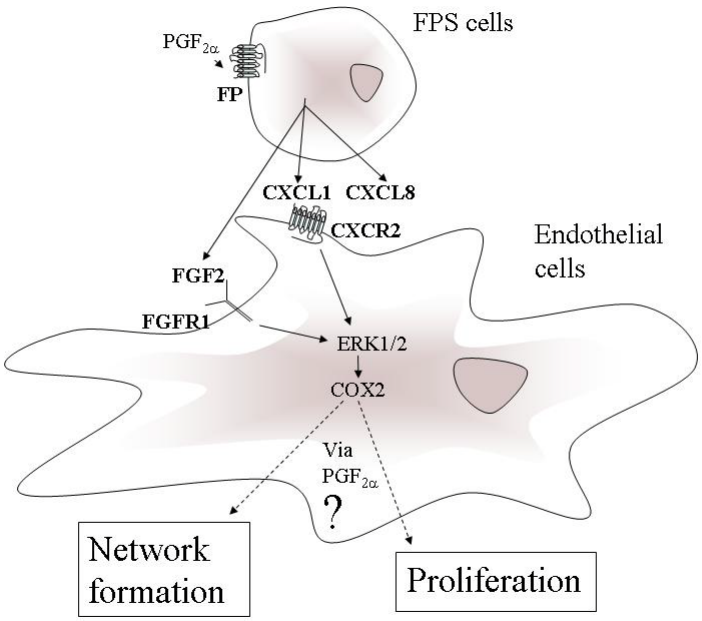


Fig. 52. A schematic representation of P CM induced signaling suggesting the involvement of COX-2 in endothelial cell network formation and proliferation is via prostaglandin $F_{2\alpha}$.

5 The role of $PGF_{2\alpha}$-FP mediated prostaglandin production on endothelial cell function.

5.1 Abstract

This chapter contains novel findings about the role of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in endothelial cell function. The direct and indirect role of $PGF_{2\alpha}$ signalling in endothelial cells was examined. Data presented here demonstrates that $PGF_{2\alpha}$ acting via the FP receptor on endothelial cells can directly regulate endothelial cell network formation but not proliferation. In the indirect role, $PGF_{2\alpha}$-FP receptor signalling in endometrial epithelial cells upregulates pro-angiogenic factors such as FGF-2. Data herein demonstrates that FGF-2 in $PGF_{2\alpha}$ conditioned medium activates ERK1/2 via its endothelial FGFR1 receptor resulting in two divergent signalling pathways, one of which leads to mTOR activation to promote endothelial cell proliferation. The second unique pathway leads to an increased production of endothelial $PGF_{2\alpha}$ which enhances endothelial cell differentiation.

5.2 Introduction

In chapter 4, it was found that P CM treatment of endothelial cells not only induced endothelial cell network formation and proliferation but also induced the secretion of prostaglandin $F_{2\alpha}$. Following on from this, the direct and indirect role of prostaglandins in regulating endothelial cell network formation and proliferation will be examined.

Prostaglandins E_2 has been shown to influence endothelial cell function in vitro and promote angiogenesis in vivo (Finetti et al., 2008; Rao et al., 2007; Tamura et al., 2006). $PGF_{2\alpha}$ is secreted by endothelial cells which express the FP receptor (Finetti et al., 2008; Taylor et al., 1987) however, its role in angiogenesis is unknown as current research has concentrated on the important role of $PGF_{2\alpha}$ in regulating vasoconstriction (Errasti et al., 2009; Wong et al., 2009). Addition of exogenous Prostaglandin E_2 to endothelial cells can increase proliferation, migration and tube

formation (Jain et al., 2008; Wang et al., 2006; Zhao et al., 2007). Jain et al. showed that exogenous PGE_2 stimulates the migration of HUVECs in an EP2 dependent manner and also showed using a matrigel plug assay that PGE_2 treatment causes an increase in CD31 stained vessels as well as an upregulation in NF-kB and Akt staining (Jain et al., 2008). In addition Jain et al., suggest that using EP receptor antagonists may be a rational way of overcoming the need for COX inhibitors (Jain et al., 2008). The use of cyclooxygenase inhibitors in therapeutic treatment met with a hurdle when in 2004 the COX-2 inhibitor celecoxib, used in a clinical trial to prevent adenomas, was found to cause cardiovascular problems including increased blood pressure (William et al., 2009). This is due to the fact that COX enzymes regulate the production of a number of prostaglandins which are essential for different mechanisms controlling normal vascular function and homeostasis (Dubois et al., 1998). Ideally, new research may be able to identify alternative COX-related therapeutic targets, such as individual prostaglandin receptors (Cha and DuBois, 2007). Targeting a specific prostaglandin pathway involved in cancer may be a way of preventing a specific downstream effect of COX, rather than ablating all COX-related functions.

Although the role of prostaglandin E_2 in angiogenesis is reasonably well studied, the role of prostaglandin $F_{2\alpha}$ has been neglected. Therefore, in this chapter the mechanisms by which $PGF_{2\alpha}$-FP epithelial signalling stimulates endothelial cell proliferation via the FGFR1-mTOR pathway and endothelial cell network formation via the endothelial FP receptor are revealed.

5.3 Results

5.3.1 The direct effect of prostaglandin $F_{2\alpha}$ and E_2 on endothelial cell function.

In order to investigate whether prostaglandin $F_{2\alpha}$ and E_2 directly regulate endothelial cell network formation and proliferation, endothelial cells were treated with exogenous $PGF_{2\alpha}$ and PGE_2 and proliferation (Fig. 53) and network assays (Fig. 54) were performed. Firstly, to examine the effects of $PGF_{2\alpha}$ on proliferation, HUVECs

were treated with 1μM $PGF_{2\alpha}$ or 0.1 μM $PGF_{2\alpha}$ for 24, 48, 72 and 96hrs. No difference was observed in proliferation rates of HUVECs treated with either concentration of $PGF_{2\alpha}$ at any of the time points tested (Fig. 53A). Similarly, treatment of HUVECs with 1μM PGE_2 or 0.1 μM PGE_2 did not significantly increase proliferation rates above that seen with control medium (EBM1% plus vehicle) alone (Fig. 53B).

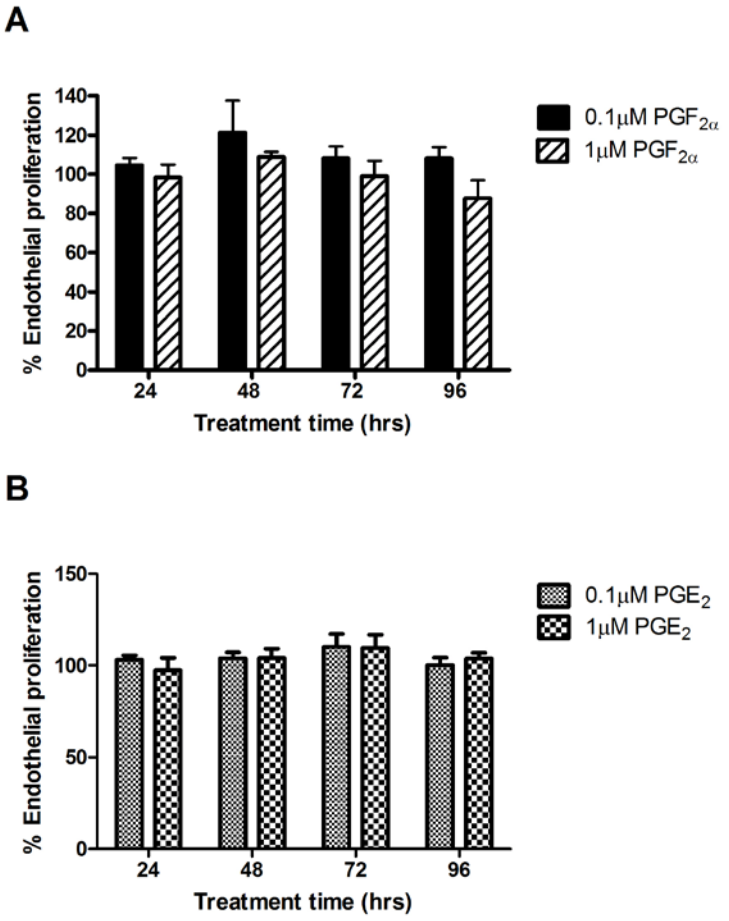


Fig. 53. HUVEC proliferation is not affected by prostaglandin E_2 and $F_{2\alpha}$ treatment. HUVECs were treated with exogenous (A) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or (B) prostaglandin E_2 (PGE_2) for 24, 48, 72 and 96hrs after which proliferation was measured by reading absorbance (490nm) following the addition of One Solution Proliferation Reagent (Promega). (* represents statistical significance $P<0.05$) Data are represented as mean ± SEM from three independent experiments.

Next to investigate the effect of $PGF_{2\alpha}$ and PGE_2 on network formation, HUVECs were treated with 1μM $PGF_{2\alpha}$ or 1μM PGE_2 for 16hrs after which network formation was assessed (Fig. 54). The addition of exogenous $PGF_{2\alpha}$ and PGE_2 significantly increased network formation above the levels seen with vehicle control medium alone (Fig. 54). This indicates that the prostaglandins signalling via their receptors may enhance network formation. To verify the role of the prostaglandin receptors, chemical antagonists of the FP receptor AL8810 and EP2 receptor AH6809 were added to the network assays in addition to the $PGF_{2\alpha}$ and PGE_2 treatment respectively. Co-treatment of HUVECs with $PGF_{2\alpha}$ and AL8810 significantly reduced network formation compared to that seen with $PGF_{2\alpha}$ alone (Fig. 54). Also, co-treatment of HUVECs with PGE_2 and AH6809 significantly reduced network formation in comparison to HUVECs stimulated by PGE_2 alone (Fig. 54). These data indicate that the FP and EP2 receptors are mediators of endothelial network formation.

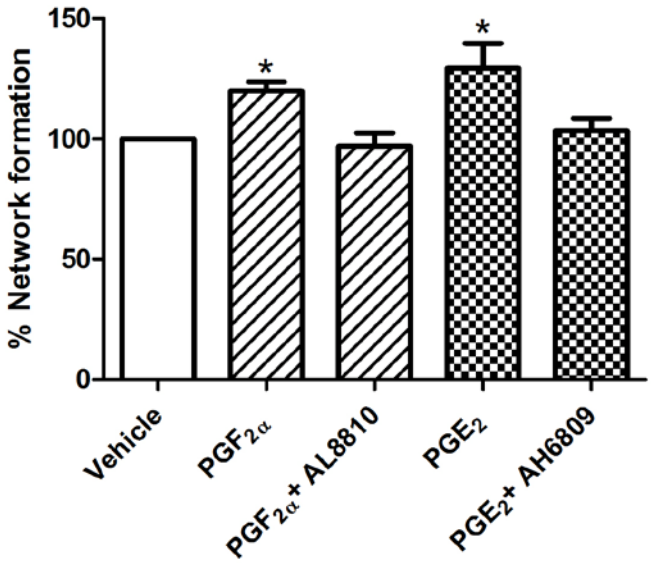


Fig. 54. Endothelial network formation with prostaglandin treatment. HUVECs were plated on growth factor reduced matrigel with or without 50μM AL8810 (FP receptor inhibitor) or 50μM AH6809 (EP2 receptor inhibitor) and incubated with 1μM exogenous $PGF_{2\alpha}$ or PGE_2 for 16hrs. The network connections/branches were counted in 10 fields per duplicate. Quantification of branches counted from at least four independent experiments. (* represents statistical significance $P<0.05$). Data are represented as mean ± SEM from five independent experiments.

5.3.2 Prostaglandin receptor mRNA expression induced by P CM in HUVECs.

Prostaglandin $F_{2\alpha}$ and prostaglandin E_2, signalling via their FP and EP2 receptors respectively, can directly stimulate HUVEC network formation and the inhibition of COX-2 prevents P CM network formation. Hence, the contribution of prostaglandin $F_{2\alpha}$ and E_2 receptors to P CM-induced endothelial cell function was investigated. Firstly, the expression of the prostaglandin receptors after P CM treatment was examined in order to identify which receptors may play a part in P CM induced endothelial cell function. HUVECs were incubated with V CM or P CM for 1, 2, 3, 4, 6, 16 and 24hrs.

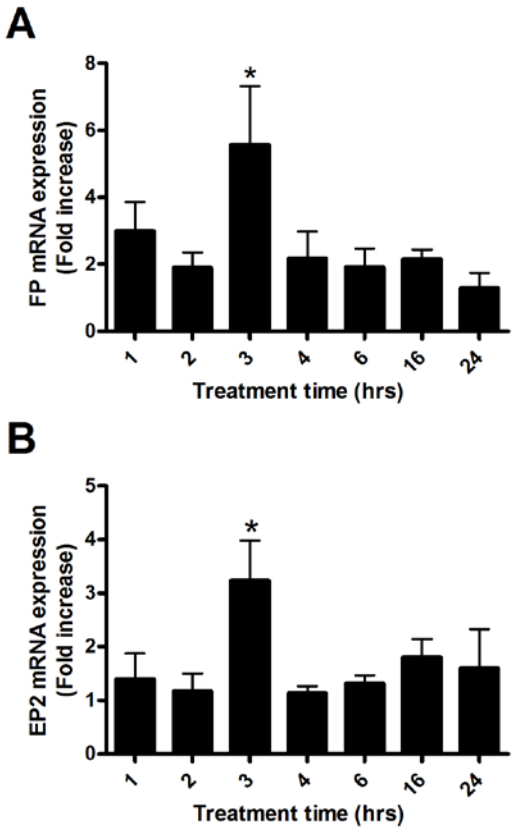


Fig. 55. Expression of prostaglandin receptors EP2 and FP after P CM treatment. HUVECs were treated with V CM or P CM for 1,2,3,4,6,16,24 hrs and mRNA expression of EP2 (A) and FP (B) was determined by RT-PCR. (* represents statistical significance from V CM; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

Treatment of HUVECs with P CM for 3hrs significantly elevated the mRNA expression of FP (Fig. 55A) and EP2 (Fig. 55B) receptors compared to V CM

treatment (P<0.05). However, no upregulation of EP1 (Fig. 56A), EP3 (Fig. 56B) or EP4 (Fig. 56C) receptor mRNA expression by P CM treatment was observed at any of the timepoints tested.

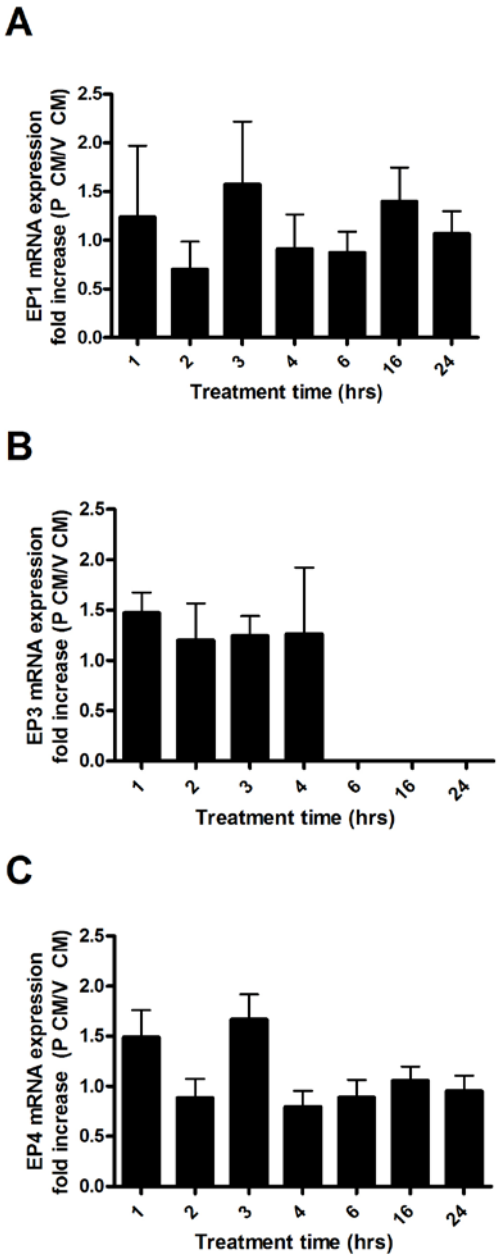


Fig. 56. Expression of endothelial cell EP receptors after P CM treatment. HUVECs were treated with V CM or P CM for 1,2,3,4,6,16,24 hrs and mRNA expression of EP1(A), EP3 (B; EP3 mRNA was not detectable after 6hrs of treatment.) and EP4 (C) was determined by RT-PCR. (* represents statistical significance from V CM; P<0.05). Data are represented as mean ± SEM from at least 5 independent experiments.

Subsequently, the signalling pathways leading to the increased expression of FP and EP2 mRNA were examined with the use of small molecule chemical inhibitors.

In chapter 4 the endothelial FGFR1 and CXCR2 receptors have been shown to be involved in the P CM induced network formation via the paracrine action of epithelial FGF2, CXCL1 and CXCL8. To examine whether FGF2-FGFR1 and CXCL1/CXCL8-CXCR2 signalling may be involved in the regulation of the FP and EP2 receptors, a small molecule chemical inhibitor against the FGFR1 receptor (SU4984) and chemical antagonist against the CXCR2 receptor (SB225002) (Fig. 57) were used. HUVECs were treated for 3hrs with V CM and P CM in the absence or presence of the chemical inhibitors after which FP (Fig. 57A) and EP2 (Fig. 57B) mRNA expression was examined. Both SU4984 and SB225002 significantly inhibited P CM-induced FP (Fig. 57A) and EP2 (Fig. 57B) mRNA expression. SU4984 and SB225002 used in combination did not have an additive affect of reducing FP (Fig. 57A) or EP2 (Fig. 57B) expression. This indicates that FGF2, CXCL1 and CXCL8, signalling via their respective receptors, are involved in the increase in FP and EP2 receptor expression induced by P CM treatment.

According to data in chapter 4 FGF2, CXCL1 and CXCL8 signalling to their receptors FGFR1 and CXCR2 respectively can activate ERK1/2 and the cyclooxygenases. In agreement, using the inhibitors, PD98059 (ERK1/2 inhibitor), Indomethacin (COX inhibitor) and NS398 (COX-2 inhibitor) in combination with P CM, significantly reduced levels of both FP (Fig. 57A) and EP2 (Fig. 57B) receptor mRNA expression compared to that seen with P CM alone ($P<0.05$). This suggests that FGF2-FGFR1 and CXCL1/CXCL8-CXCR2 induced endothelial cell signalling regulates endothelial cell PG receptor expression.

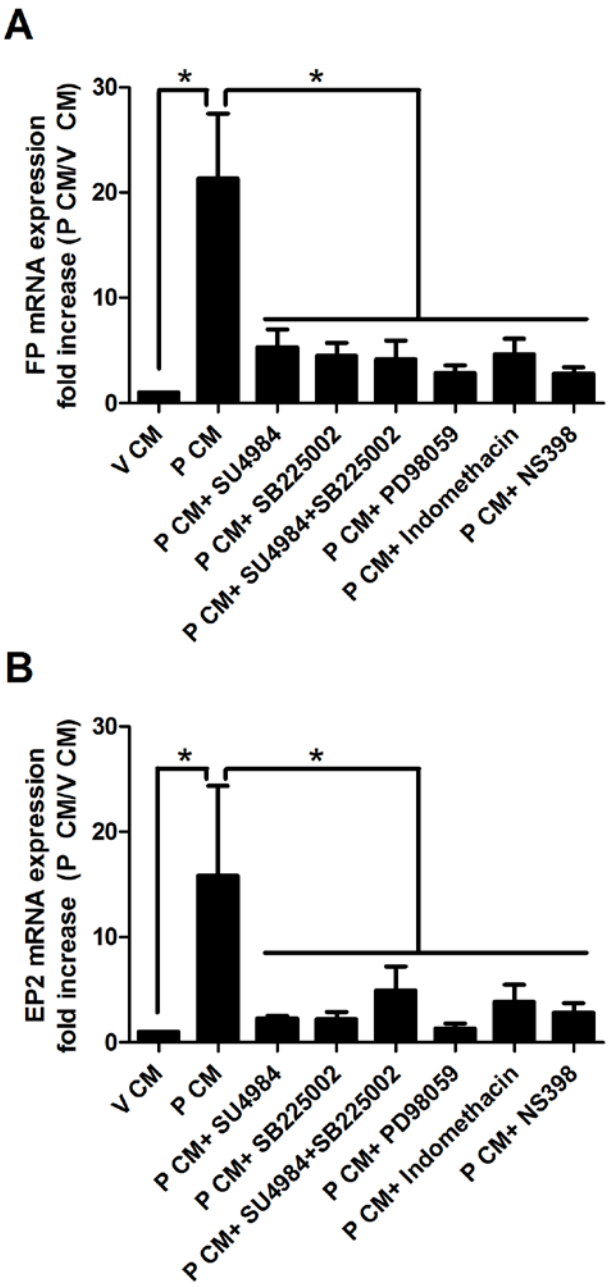


Fig. 57. FP and EP2 expression regulated by P CM induced signalling. HUVECs were treated for 3hrs with V CM or P CM in the absence/presence of SU4984 (FGFR1 tyrosine kinase inhibitor), CXCR2 (SB225002), PD98059 (ERK1/2 inhibitor), Indomethacin (COX inhibitor) or NS398 (COX2 inhibitor) and mRNA expression of FP (A) and EP2 (B) was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

5.3.3 The role of the FP and EP2 receptors in P CM-induced endothelial cell function

Previously in this chapter, endothelial cell network formation (Fig. 54) but not proliferation (Fig. 53) was found to be stimulated by exogenous $PGF_{2\alpha}$ and PGE_2. To verify the role of the FP and EP2 receptors in P CM induced endothelial cell function, network assays and proliferation assays were performed firstly using inhibitors of the FP and EP2 receptors. Treatment of HUVECs with P CM and antagonists of FP and EP2 receptors, AL8810 and AH6809 respectively, significantly reduced network formation compared to that seen with P CM treatment alone (Fig. 58A, $P<0.05$). Co-treatment of HUVECs with P CM and AL8810 and AH6809 had a slight but not significant additive affect on preventing endothelial cell network formation (Fig. 58A). In contrast, P CM induced proliferation was not inhibited by the addition of AL8810 or AH6809 even when used in combination (Fig. 58B).

As shown in chapter 4, only $PGF_{2\alpha}$ secretion was increased by P CM treatment. This suggests a prominent role for $PGF_{2\alpha}$-FP autocrine signalling in P CM-induced endothelial cell network formation. As the role of the FP receptor in endothelial cell network formation is a novel finding, to support the chemical inhibitor data, two short hairpin RNA (shRNA) constructs of the FP receptor, sh306 and sh478 in adenovirus, were used to knockdown FP receptor signalling (section 2.9.3.2). Once the FP receptor knockdown was verified (see section 2.9.3.2), HUVECs were infected with scrambled control virus (scr), sh306 and sh478 and network formation and proliferation rates were assessed. HUVECs infected with the control scr virus showed an increase in network formation with P CM treatment compared to V CM treatment (Fig. 59A, $P<0.05$). In contrast, network formation in sh306 and sh478 infected cells did not increase after P CM treatment compared to V CM treatment (Fig. 59A). This suggests that P CM induced endothelial cell network formation requires the endothelial FP receptor. In support of the results collected with use of

AL8810, knocking down the FP receptor with sh306 and sh478 did not prevent P CM-mediated endothelial cell proliferation (Fig. 59B). Therefore, it is likely that endothelial $PGF_{2\alpha}$-FP signalling is not involved in P CM induced proliferation.

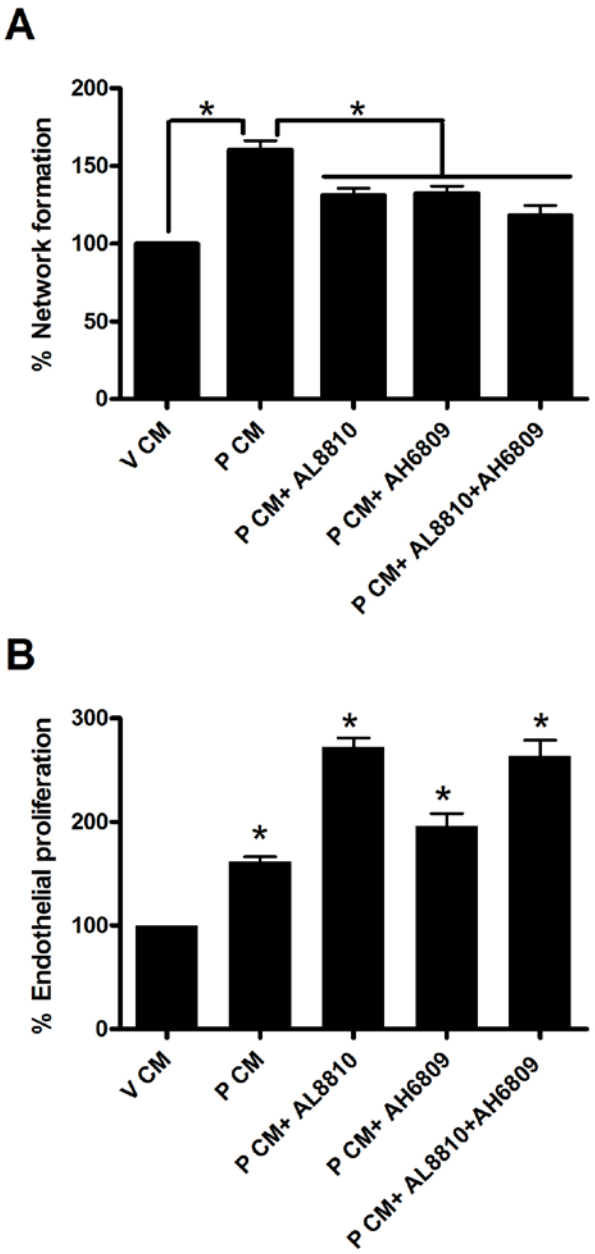


Fig. 58. The role of FP and EP2 in P CM-induced endothelial cell function. A, HUVECs were seeded on matrigel, with or without 50μM AL8810 (FP receptor inhibitor) or AH6809 (EP2 receptor inhibitor), and the network assay was performed with V CM and P CM. B, HUVECs were treated with V CM and P CM plus or minus AL8810 or AH6809 for 96hrs after which proliferation was measured by reading absorbance (490nm) following the addition of One Solution Proliferation Reagent (Promega). (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from four independent experiments.

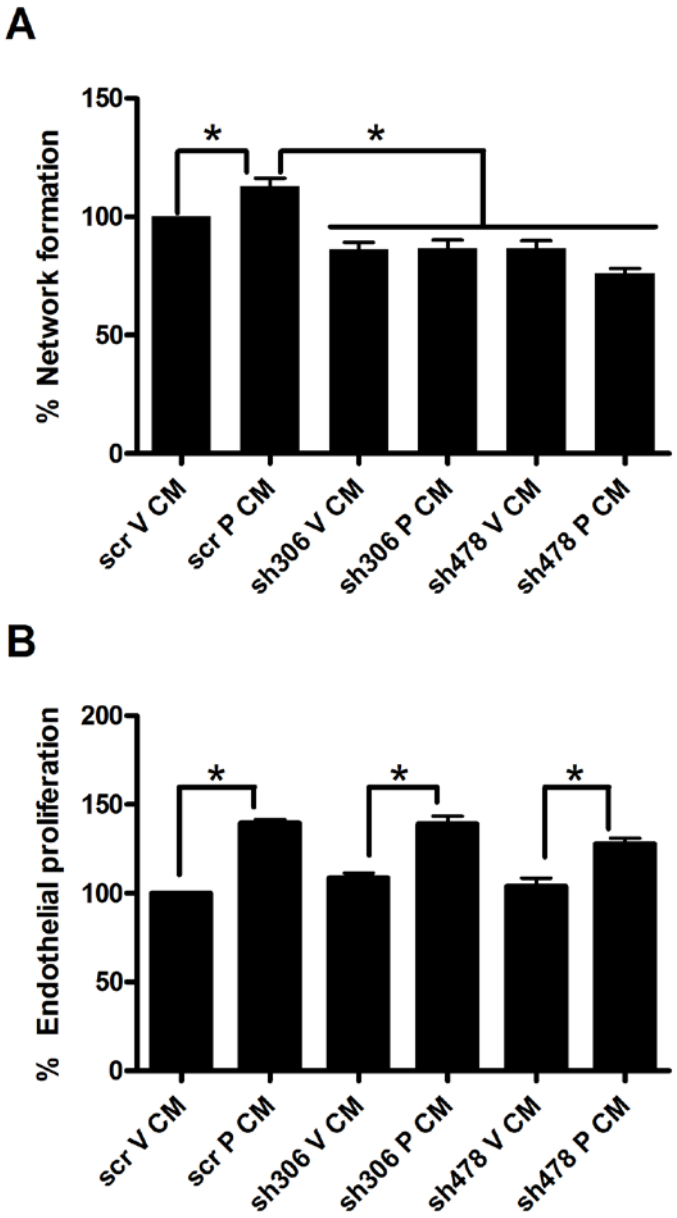


Fig. 59. Decreasing FP receptor with shRNA expression affects endothelial cell network formation. HUVECs were infected with scrambled adenovirus (scr) or short hairpin adenovirus targeted against the FP receptor (sh306 and sh478) and treated with V CM or P CM. Endothelial network formation (A) and proliferation (B) were determined as described in the methods. (* represents statistical significance $P<0.05$) Data are represented as mean ± SEM from five independent experiments.

5.3.4 The regulation of endothelial cell proliferation by proangiogenic factor signalling.

The previous data in this chapter shows that endothelial $PGF_{2\alpha}$ plays a role in promoting P CM-induced endothelial cell network formation but is not involved in endothelial cell proliferation. This leads to the suggestion that it is the growth factors in the conditioned medium, identified in chapter 4, that are controlling P CM induced proliferation independently of endothelial prostaglandin secretion. In support of this, HUVECs treated with P CM immunoneutralised with FGF2, CXCL1, CXCL8 all showed reduced proliferation rates compared to cells treated with P CM alone (Fig. 60A). Equally, recombinant FGF2, CXCL1 and CXCL8 can all stimulate endothelial cell proliferation above that seen with basal cell medium control (EBM1%). FGF2 has been shown to promote the secretion of VEGF-A facilitating tumour growth and angiogenesis, in vivo using an endometrial cancer xenograft model (Giavazzi et al., 2003). This demonstrates that the coordination of growth factors is needed for angiogenesis. To shed some light on the mechanism by which growth factors in the P CM are involved in endothelial cell proliferation, the pathway stimulated by fibroblast growth factor 2 was chosen as FGF2 is a potent growth factor involved in proliferation of many cell types including endothelial cells (Presta et al., 2005; Sales et al., 2007; Seghezzi et al., 1998). Using chemical inhibitors of FGFR1, PP2, ERK1/2 shown in Fig. 60B, as found in chapter 4, the P CM induced proliferation, was found to be regulated by FGF2 signalling to FGFR1 and activating ERK1/2 via c-Src. Therefore, the pathways controlling network formation and proliferation could diverge after ERK1/2 activation or else before via another alternative pathway from FGFR1.

One pathway that has been implicated in cellular proliferation is the mTOR pathway (Bjornsti and Houghton, 2004). mTOR can be activated via the PI3K pathway therefore, chemical inhibitors of mTOR (rapamycin) and PI3K (LY294002 and wortmannin) were used to examine the role of the PI3K-mTOR pathway in P CM induced proliferation (Fig. 61). Interestingly none of the inhibitors, rapamycin,

LY294002 or wortmannin inhibited endothelial cell network formation (Fig. 61A). In contrast, rapamycin was found to inhibit P CM-induced proliferation but neither LY294002 nor wortmannin had an effect on P CM-induced endothelial cell proliferation (Fig. 61A).

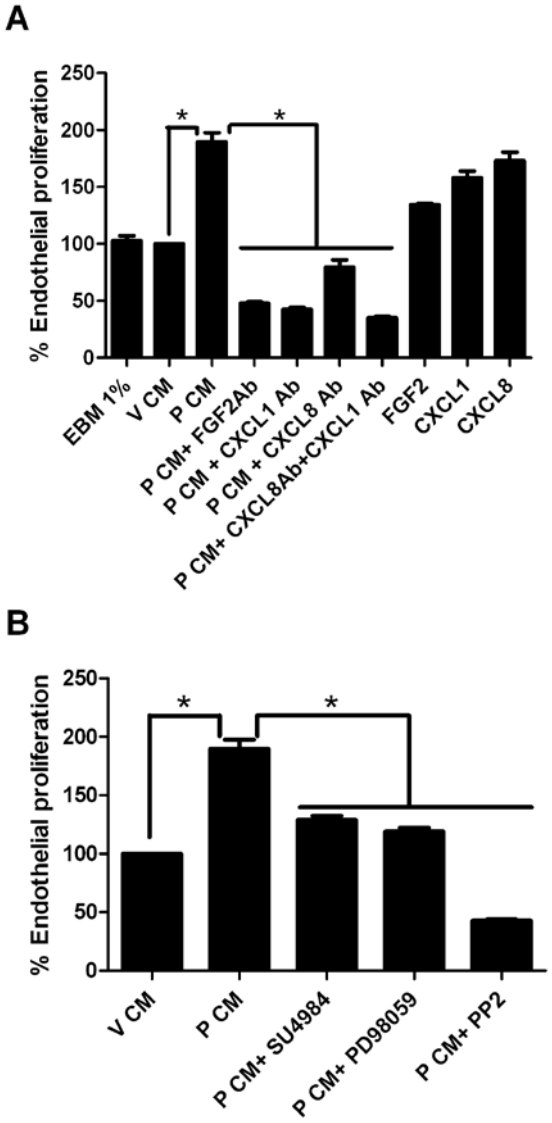


Fig. 60. Growth factors in P CM control endothelial cell proliferation. A, HUVECs were treated with V CM, P CM or P CM immunoneutralised with antibodies for FGF2, VEGFA, CXCL1, CXCL8, IL6 or PDGFA. FGF2, VEGFA, CXCL1 and CXCL8 were used as a positive control. B, HUVECs were treated with V CM, P CM, P CM plus SU4984 (FGFR1 inhibitor), PD98059 (ERK1/2 inhibtor) or PP2 (c-Src inhibitor). After 96hrs, proliferation was assessed after the addition of One Solution Reagent (Promega) and absorbance was measured with a spectrophotometer at 490nm. Data from at least three independent experiments represented at mean ± SEM. (* represents statistical significance $P<0.05$).

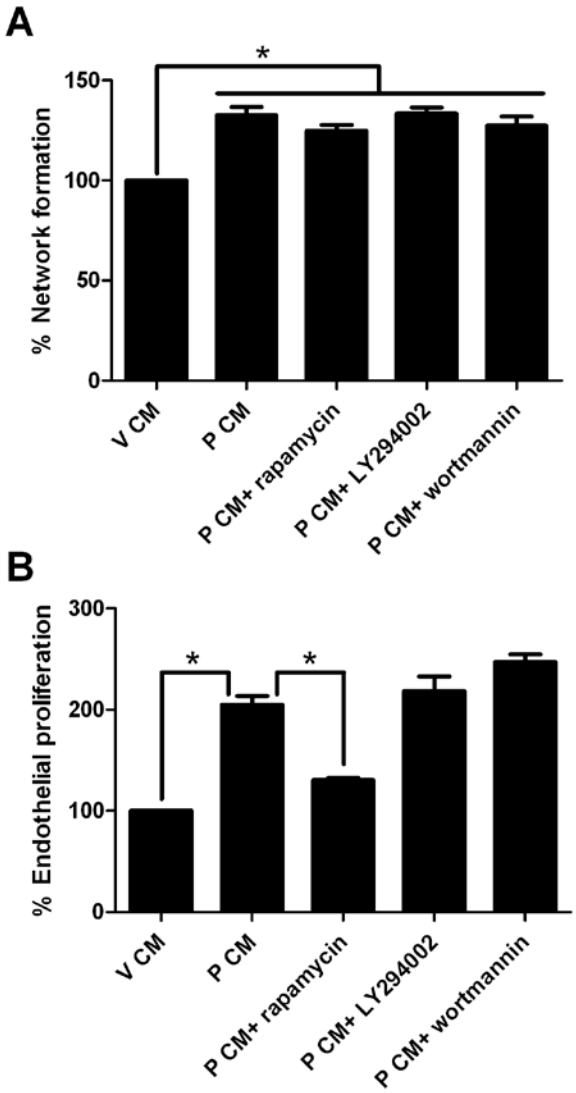


Fig. 61. The role of PI3K-mTOR pathway in P CM induced endothelial cell function. A, HUVECs were seeded on matrigel containing rapamycin (mTOR inhibitor), LY294002 (PI3K inhibitor) or wortmannin (PI3K inhibitor) and the network assay was performed. B, HUVECs were treated with V CM and P CM plus or minus rapamycin, LY294002 or wortmannin for 96hrs afterwhich proliferation was measured by reading absorbance (490nm) following the addition of One Solution Proliferation Reagent (Promega). (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of at least three independent experiments.

To confirm whether PI3K plays a role in P CM signalling, HUVECs were treated with V CM and P CM for 0, 5, 10, 15, 20 and 30 minutes (Fig. 62). Western blot analysis of the PI3K regulatory subunit p85 alpha, which is activated by FGFR1 in murine brain endothelial cells (IBECs) (Cross et al., 2002), showed that P CM treatment did not induced PI3K phosphorylation at any of the time points tested compared to V CM treatment (Fig. 62). These data along with that of Fig. 61B indicates that activation of the PI3K regulatory subunit p85 is not involved in P CM induced endothelial cell proliferation.

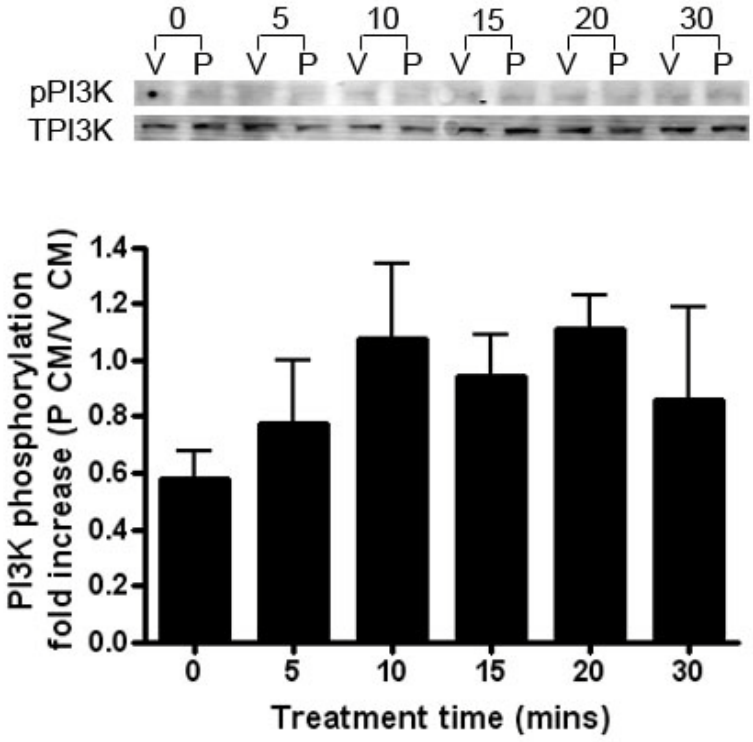


Fig. 62. PI3K p85α phosphorylation is not induced by P CM treatment. HUVECs were treated with V CM (V) or P CM (P) for 0,5,10,15,20 and 30 minutes. Cell lysates were subjected to immunoblot analysis using antibodies against phosphorylated PI3K (top panel) and total PI3K (bottom panel). A representative Western blot is displayed with a graph of semi-quantitative analysis of PI3K phosphorylation determined as described in Materials and Methods. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of three independent experiments.

To confirm the position of rapamycin in the signalling cascade resulting from P CM treatment and leading to endothelial cell proliferation, HUVECs were treated with V CM, P CM and P CM and rapamycin for 10 minutes and ERK1/2 phosphorylation was assessed by immunoblot analysis (Fig. 63). Rapamycin did not inhibit P CM induced ERK1/2 phosphorylation indicating that it is downstream of ERK1/2 activation (Fig. 63).

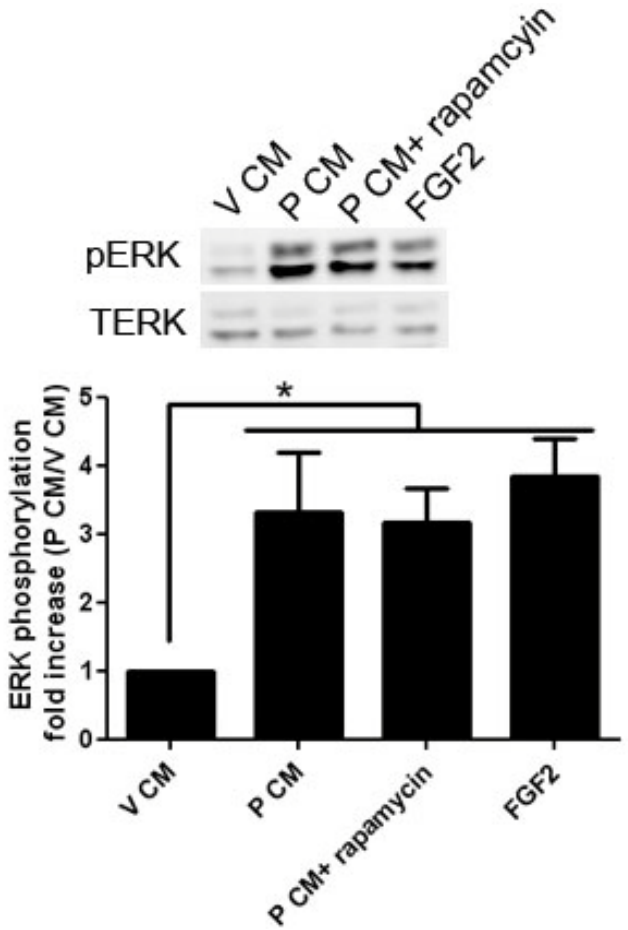


Fig. 63. P CM induced ERK1/2 phosphorylation is independent of mTOR activation. HUVECs were treated for 10minutes with V CM, P CM or P CM and rapamycin. FGF2 was used as a positive control for ERK1/2 phosphorylation. Cell lysates were subjected to immunoblot analysis using antibodies against phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel). A representative Western blot is displayed with a graph of semi-quantitative analysis of ERK1/2 phosphorylation determined as described in Materials and Methods. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of three independent experiments.

5.3.5 Confirmation of the FGF2 induced network formation and proliferation pathways.

In order to confirm that the divergent pathways involved in P CM induced HUVEC network formation and proliferation are mediated by FGF2, HUVECs were treated with recombinant FGF2 in the absence or presence of the c-Src inhibitor PP2, the ERK1/2 inhibitor PD98059 and the mTOR inhibitor rapamycin (Fig. 64).

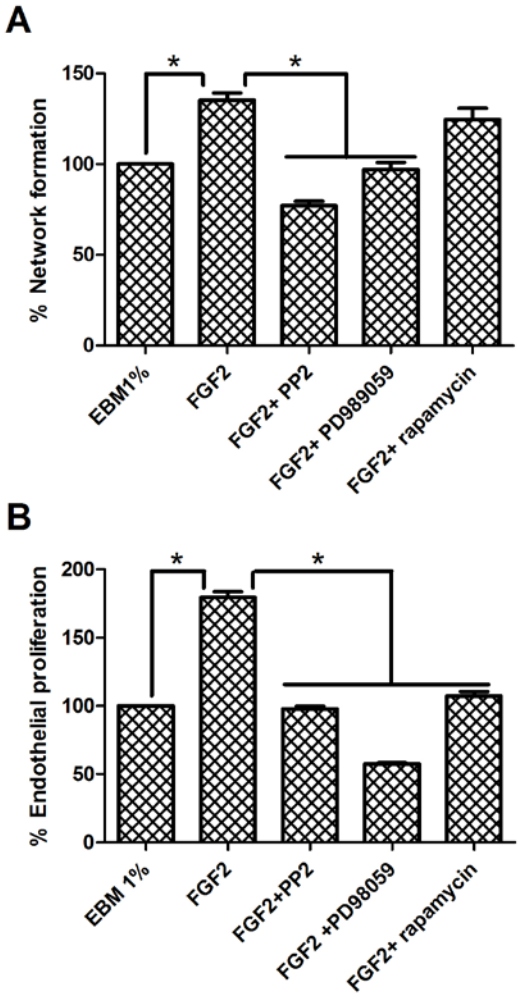


Fig. 64. The signalling pathways involved in FGF2 induced endothelial cell function. A, Endothelial network formation and B, proliferation in HUVECs treated with vehicle or recombinant FGF2 protein (50ng/ul) in the absence/presence of PD98059 (ERK1/2 inhibitor), PP2 (c-Src inhibitor) or rapamycin (mTOR inhibitor). (* represents statistical significance $P<0.05$) Data are represented as percentage increase compared to EBM1% and presented as mean ± SEM of at least three independent experiments.

Recombinant FGF2 significantly increased endothelial cell network formation (Fig. 64A, $P<0.05$) and proliferation (Fig. 64B, $P<0.05$). Addition of PP2 and PD98059 to recombinant FGF2 treatment inhibited both network formation (Fig. 64A, $P<0.05$) and proliferation (Fig. 64B, $P<0.05$) however, rapamycin significantly inhibited only FGF2 induced proliferation (Fig. 64B, $P<0.05$) and had no significant effect on network formation (Fig. 64A), similar to the results seen for CM (Fig. 61).

Previous studies have been uncertain about whether or not c-Src is required for FGF2 induced endothelial cell ERK1/2 activation as Kilarski et al. showed using immortalised brain endothelial cells (IBECs) that overexpressing an inhibitor of Src did not prevent ERK1/2 phosphorylation by FGF2 (Kilarski et al., 2003). As both c-Src and ERK1/2 are involved in P CM- and FGF2-induced proliferation and c-Src inhibits P CM induced ERK1/2 activation (as shown in chapter 3), the effect of the c-Src inhibitor, PP2 on recombinant FGF2 induced ERK1/2 activation in HUVECs was assessed (Fig. 65).

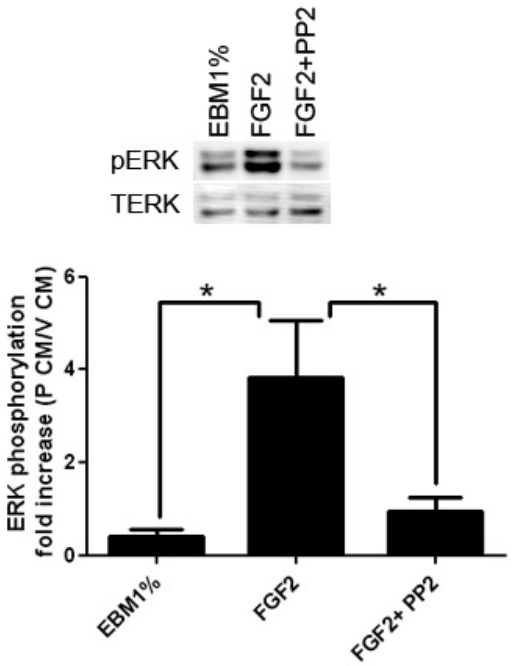


Fig. 65. c-Src is involved in FGF2 induced ERK1/2 phosphorylation. HUVECs were treated for 10 minutes with EBM1%, FGF2 (50µg/µl) or FGF2 plus PP2. Cell lysates were subjected to immunoblot analysis using antibodies against phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel). A representative Western blot is displayed with a graph of semi-quantitative analysis of ERK1/2 phosphorylation determined as described in

Materials and Methods. (* represents statistical significance; P<0.05). Data are represented as mean ± SEM of three independent experiments.

Co-treatment of HUVECs with FGF2 and PP2 significantly reduced ERK1/2 activation seen with recombinant FGF2 treatment alone (Fig. 65, P<0.05). This confirms that c-Src is involved in the FGF2-FGFR1 induced activation of ERK1/2 in both P CM and recombinant FGF2 stimulated pathways.

5.4 Discussion

At the end of chapter 4, data showed that $PGF_{2\alpha}$ and PGE_2 were biosynthesised by HUVECs however only $PGF_{2\alpha}$ secretion was increased, along with cyclooxygenase-2 expression, by P CM treatment. This suggests that prostaglandin $F_{2\alpha}$ may be important in the regulation of endothelial cell function.

In order investigate the direct effects of $PGF_{2\alpha}$ and PGE_2, HUVECs were treated with exogenous $PGF_{2\alpha}$ or PGE_2 and network formation and proliferation were examined. Exogenous prostaglandin $F_{2\alpha}$ treatment was compared with prostaglandin E_2 treatment as previous data has shown that PGE_2 can influence endothelial cell network formation, proliferation and migration (Jain et al., 2008; Wang et al., 2006; Zhao et al., 2007). A study by Tamura et al. showed in HUVECs, that exogenous PGE_2 induced network formation and implicated PGE_2 and COX-2 in the regulation of VEGF-A induced network formation (Tamura et al., 2006). A recent study by Finetti et al. showed that PGE_2 treatment of endothelial cells causes EP3 and c-Src activation followed by FGF2-FGFR1-ERK1/2 signalling which enhances endothelial cell migration (Finetti et al., 2008). Also previous research has shown that endothelial cell proliferation inhibited by COX-2 siRNA was partially rescued by addition of PGE_2 (Zhao et al., 2007). However, in a study investigating the role of EP4 receptor in endothelial cell network formation, proliferation, and migration, PGE_2 treatment did not increase murine endothelial cell proliferation in EP4-null cells or the control cells (Rao et al., 2007). Data presented in this thesis showed that neither exogenous $PGF_{2\alpha}$ nor exogenous PGE_2 could induce endothelial cell

proliferation at the concentrations tested. This in vitro investigation does not rule out the fact that these prostaglandins may be involved in proliferation in vivo. For example, Leahy et al. found that the addition of COX-2 inhibitor celecoxib to an FGF2-induced mouse corneal model of angiogenesis inhibited endothelial cell proliferation which was attributed to a decrease in PGE_2 production (Leahy et al., 2002). However, as it may be difficult to distinguish between endothelial cell proliferation and migration using in vivo models, perhaps the action of celecoxib could have also been inhibiting the chemotactic role of prostaglandins in stimulating endothelial cell migration (Rao et al., 2007).

In accordance with previous data, exogenous PGE_2 was found to stimulate HUVEC network formation in an EP2 dependent manner. In vivo data by Jain et al. has shown that the EP2 receptor is involved in PGE_2 stimulated angiogenesis (Jain et al., 2008). Additionally, this chapter presents the novel finding that exogenous $PGF_{2\alpha}$ stimulates endothelial cell network formation in an FP dependent manner since the FP inhibitor significantly reduced $PGF_{2\alpha}$-induced endothelial cell network formation. This suggests that $PGF_{2\alpha}$ present in the P CM could be acting directly to promote endothelial cell network formation in a paracrine and autocrine manner via the endothelial FP receptor.

To assess the direct role of the prostaglandins and their receptors in P CM-induced endothelial cell function, the levels of prostaglandin receptors after P CM treatment were investigated. All the EP receptors 1-4 have been shown to be involved in tumourigenesis, angiogenesis or endothelial cell function (Amano et al., 2003; Dorsam and Gutkind, 2007; Jain et al., 2008; Kamoshita et al., 2006). In this study, the FP and EP2 receptors were upregulated after P CM treatment indicating these receptors are involved in the P CM-induced network formation and proliferation. Data herein demonstrates that the FP and EP2 receptors, the receptors for $PGF_{2\alpha}$ and PGE_2 respectively, are regulated in a manner requiring FGF2-FGFR1 or CXCL1/CXCL8-CXCR2 signalling, the activation of ERK1/2 and cyclooxygenases 1 and 2. In line with these data, a previous study by Kuwano et al. showed in a

mouse corneal model that the regulation of angiogenesis by cytokine signalling involved increasing COX-2 resulting in elevated prostaglandin E_2 signalling through endothelial EP2 and EP4 receptors (Kuwano et al., 2004). Additionally, prostaglandin $F_{2\alpha}$ secretion was shown to be induced via COX-2 after IL-1 treatment of endometrial stromal cells (Kawano et al., 2001) and TNFα stimulates $PGF_{2\alpha}$ secretion via ERK1/2 activation in bovine luteal cells (Sakumoto R et al., 2000).

Using the in vitro indicators of angiogenesis, endothelial cell network assays and proliferation assays, data in this thesis demonstrate that P CM-induced endothelial cell network formation is regulated by $PGF_{2\alpha}$ signalling through the FP receptor. In contrast, P CM-induced endothelial cell proliferation was not inhibited by the FP receptor antagonist suggesting that, as seen with the exogenous $PGF_{2\alpha}$, $PGF_{2\alpha}$-FP signalling does not directly regulate endothelial cell proliferation. The apparent increase in proliferation seen with the addition of AL8810, is likely due to the fact that AL8810 can act as an agonist when in excess (Griffin et al., 1999). The evidence for the role of the FP receptor in P CM-induced endothelial cell network formation was further supported by use of a specific FP receptor short hairpin RNA in an adenoviral delivery system for targeted ablation of endothelial FP receptor. Reducing the FP receptor levels in endothelial cells, reduced endothelial cell network formation induced by P CM but had no effect on endothelial cell proliferation.

The EP2 receptor antagonist also significantly inhibited P CM-induced network formation. This suggests that even though the PGE_2 secretion is not upregulated by P CM, the upregulation of the EP2 receptor is enough to contribute to the P CM-induction of endothelial cell network formation. No evidence was found here to indicate that PGE_2-EP2 receptor signalling is involved in P CM-induced proliferation. One interesting explanation for the role of the EP2 receptor in the P CM-induced network formation is the possibility that $PGF_{2\alpha}$ could act through the EP receptors. For example, in a recent study involving mast cells which don't express the FP receptor, $PGF_{2\alpha}$ treatment was found to increase cytokine secretion in an EP receptor dependent manner (Kaneko et al., 2008). The affinity of $PGF_{2\alpha}$ for the EP2

receptor is Ki 119nM, much less than that for the FP receptor (Abramovitz et al., 2000), but enough to promote robust cellular signalling via the EP2 receptor. In accordance, in endometrial epithelial cells, PGE_2 can signal via the FP receptor to enhance COX-2 expression (Sales et al., 2008b) demonstrating the flexibility of prostaglandins for PG receptors.

Alternatively, another explanation for the role of the EP2 receptor in the P CM-induced network formation is that conventional intracellular prostaglandins may signal through PG receptors expressed on the nuclear membrane. All the prostaglandin E_2 receptors EP1-4 are present on the nuclear membrane in cerebral microvascular endothelial cells and the EP1 receptor has been shown to be able to induce Ca^{2+} signalling when situated on the nuclear membrane indicating that it is functional (Bhattacharya et al., 1998). Another study showed that agonist activation of perinuclear EP3 caused an increase in nuclear eNOS expression indicating that nuclear prostaglandin receptors can regulate gene expression (Gobeil et al., 2002). In addition, COX-2 has been shown to be localised to the perinucleus and nucleus in a stimulation regulated manner in HUVECs (Parfenova et al., 2001). It is possible that due to the proximity of intracellular COX and PGE_2 to nuclear EP receptors, an increase in nuclear EP receptor number could efficiently enhance PGE_2 stimulated gene transcription, even in the absence of elevated PGE_2 secretion.

The data in chapter 4 showed that the COX inhibitors inhibit endothelial cell proliferation however, as we found no involvement in the EP2 or FP receptors in endothelial cell proliferation it is possible that the COX-dependent endothelial proliferation is reliant on a different COX product. One possibility is that prostacyclin is the COX product involved in endothelial cell proliferation. The IP receptor has been implicated in the regulation of endometrial angiogenesis (Smith et al., 2006). Prostacyclin treatment can induce angiogenesis and vascular permeability (Pola et al., 2004). For example, in guinea pigs, intradermal injection of VEGF-A, but not FGF2, upregulated PGI_2 expression and increased vascular permeability both of which could be inhibited by the addition of the COX inhibitor Indomethacin

(Murohara et al., 1998). An alternative COX product is thromboxane, TxA_2 which signals through the TxA2 receptor expressed on endothelial cells. Inhibition of the TxA2 receptor inhibits IL1β induced mouse corneal angiogenesis (Kuwano et al., 2004). Further investigations are required to elucidate the roles of prostacyclin and thromboxane in endometrial adenocarcinoma angiogenesis.

Prostaglandin $F_{2\alpha}$ and the FP receptor were found to directly regulate endothelial cell network formation however, they were not found to directly regulate endothelial cell proliferation. Therefore, the indirect role of $PGF_{2\alpha}$ in regulating HUVEC proliferation was investigated further as $PGF_{2\alpha}$-FP signalling in epithelial cells causes the secretion of growth factors. These growth factors (as shown in chapter 4) FGF2, CXCL1 and CXCL8, are responsible for endothelial cell proliferation and network formation but the intracellular pathways regulating proliferation have yet to be elucidated. Although, immunoneutralisation of FGF2, CXCL1 and CXCL8 all reduced P CM dependent proliferation, FGF2 was chosen to investigate the P CM-induced proliferation, as it is a potent growth factor in many cell types and there is an abundance of in vivo data supporting its role in angiogenesis (Presta et al., 2005). FGF2 binds FGFR1 and signals through c-Src and ERK1/2 to regulate P CM-induced proliferation. As shown in chapter 4, both proliferation and network formation involve the FGF2 activation of ERK1/2 therefore it is likely that the divergent proliferation pathway occurs after this signalling intermediate or before via an alternative proliferation pathway activated by FGFR1.

One possible proliferation pathway is the phosphatidylinositol 3-kinase (PI3K) -Akt-rapamycin (mTOR) pathway (Vivanco and Sawyers, 2002). FGF2 signals to the PI3K pathway in endothelial cells (Cross et al., 2002; Sulpice et al., 2002), which can activate mTOR (Bjornsti and Houghton, 2004), to promote endothelial cell proliferation (Zheng et al., 2008). Data presented in this chapter show that mTOR is involved in P CM-induced proliferation but not network formation. This is in agreement with the observations of Kanda et al., who demonstrated in murine brain endothelial cells that FGF2-induced endothelial network formation is not dependent

on activation of the mTOR pathway (Kanda et al., 1997). Interestingly, neither P CM-induced proliferation nor network formation involved the PI3K pathway as inhibitors of PI3K did not affect these endothelial functions. To confirm the absence of PI3K activation, PI3K phosphorylation was investigated and was not found to be activated by P CM treatment. The p85α regulatory subunit investigated was the regulatory subunit which can be phosphorylated by FGF2 signalling through the FGFR1 tyrosine kinase as well as other growth factors signalling through their receptors (Gesbert et al., 1998; Ong et al., 2001; Wu et al., 2001). Recent studies into the role of PI3K in tumourigenesis have involved the investigation of the p110 catalytic subunits but evidence suggests that these subunits are activated by GPCRs rather than tyrosine kinases (Jia et al., 2008). As shown in chapter 4, ERK1/2 phosphorylation was not inhibited by treatment with a PI3K inhibitor and these data together with the fact that PI3K inhibitors had no effect on endothelial cell proliferation indicate that P CM induced proliferation is independent of PI3K but dependent on mTOR signalling. Similar results were found by Kanda et al. in murine brain capillary endothelial cells who showed that the cell cycle regulator, p70S6kinase, was activated by mTOR independently of PI3K (Kanda et al., 1997). The opposite scenario also occurs after angiotensin II treatment in rat smooth muscle cells where proliferation is PI3K dependent but mTOR independent indicating that proliferative responses are cell type and stimulus specific (Dugourd et al., 2003). Data herein demonstrates that mTOR is downstream of ERK1/2 activation by P CM as the mTOR inhibitor had no effect on ERK1/2 phosphorylation.

To confirm that FGF2 in the P CM can stimulate this pathway to proliferation, endothelial cell proliferation stimulated by recombinant FGF2 was shown to involve the activation of c-Src, ERK1/2 and mTOR. In contrast, inhibiting mTOR activity had no effect on recombinant FGF2-induced endothelial cell network formation. The involvement of c-Src in FGF2-induced ERK1/2 activation was unclear because one study showed, in murine brain endothelial cells, that FGFR1 activation of ERK1/2 was independent of c-Src (Cross et al., 2002) but other studies have shown that c-Src is needed for FGF2-induced HUVEC proliferation (Deo et al., 2002) and endothelial

cell network formation (Klint et al., 1999). To clarify the role of c-Src in ERK1/2 activity in HUVECs, the c-Src inhibitor, PP2, was used in combination with recombinant FGF2 to demonstrate that FGF2 induced ERK1/2 phosphorylation is dependent on c-Src activity. This is in accordance with research by Kho et al. showing that c-Src and ERK1/2 are essential for vascular lumen formation in vitro (Koh et al., 2009).

Following ERK1/2 activation, mTOR has been shown to be regulated via the tuberous sclerosis complex 1 and 2 (TSC1/2 also called Hamartin and Tuberin) (Guertin and Sabatini, 2007; Lee and Hung, 2007). Phosphorylation of TSC2 by ERK1/2 results in its dissociation from TSC1 and its subsequent degradation via the ubiquitin pathway. This inactivates the inhibitory effect of TSC1/2 on the mTOR pathway and allows cellular proliferation to proceed (Drakos et al., 2008).

Taken together, these data confirm that the P CM-induced proliferation can be mediated by FGF2 in a c-Src, ERK1/2, mTOR dependent pathway. Endothelial cell proliferation and network formation pathways are both stimulated by FGF2 signalling to FGFR1 but diverge after ERK1/2 activation. After which, the upregulation of COX-2 enhances P CM-induced endothelial cell network formation by increasing $PGF_{2\alpha}$ signalling via endothelial cell FP receptor (see Fig. 66 for a schematic).

In summary, this chapter demonstrates that pro-angiogenic factor FGF-2, produced in endometrial epithelial cells via the $PGF_{2\alpha}$-FP receptor signalling, can regulate the endothelial cell functions of differentiation and proliferation, required for angiogenesis. Epithelial FGF-2 binds to endothelial FGFR1 receptors and activates ERK1/2 resulting via two divergent signalling pathways, one of which leads to mTOR activation to promote endothelial cell proliferation. The second unique pathway leads to an increased production of endothelial $PGF_{2\alpha}$ which directly enhances endothelial cell differentiation.

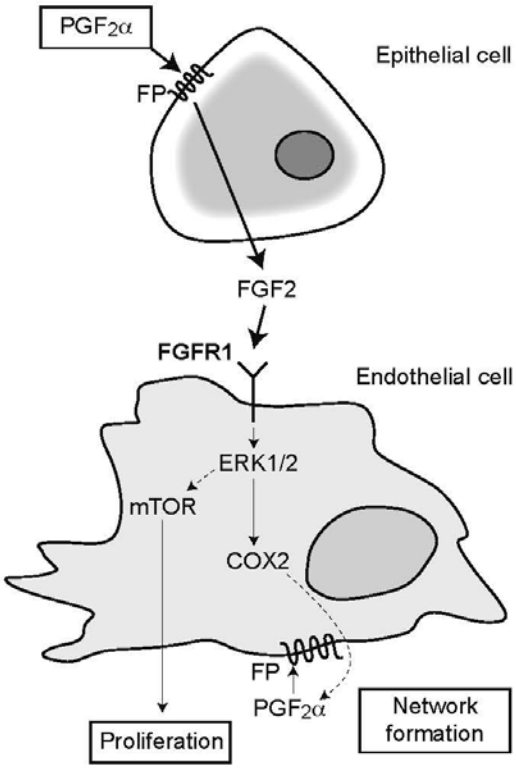


Fig. 66. Schematic representation of the divergent pathways regulating P CM-induced endothelial cell network formation and proliferation.

6 The role of antiangiogenic ADAMTS1 and RCAN-1 in P CM-induced endothelial cell function.

6.1 Abstract

Data presented in this chapter demonstrates P CM-induced endothelial cell network formation and proliferation is regulated by antiangiogenic proteins known as a disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1) and regulator of calcineurin 1-4 (RCAN1-4). ADAMTS1 overexpression significantly inhibited P CM-induced network formation and proliferation. RCAN1-4 overexpression in HUVECs inhibited P CM-induced endothelial cell network formation and proliferation possibly through a dual mechanism involving the inhibition of angiogenic cytokine CXCL8 expression and the upregulation of antiangiogenic protein ADAMTS1. In contrast, RCAN1-4 deficient HUVECs exhibited an increase in P CM-induced network formation but a decrease in P CM-induced endothelial cell proliferation occurs possibly resulting from signalling to apoptotic pathways. These data implicate ADAMTS1 and RCAN1-4 as regulators of vascular remodelling in endometrial pathologies where the FP receptor is aberrantly expressed.

6.2 Introduction

Endothelial cell function and angiogenesis are tightly regulated by a balance between proangiogenic factors and antiangiogenic factors. Antiangiogenic factors such as thrombospondin and endostatin have been shown to counteract proangiogenic VEGF-A-induced endothelial cell function in vitro and angiogenesis in vivo (Kyriakides et al., 2001; Lee et al., 2006; Margosio et al., 2008) (Abdollahi et al., 2004; Schumacher et al., 2007). In the preceding chapters, the role of proangiogenic FGF2, CXCL1 and CXCL8 in P CM-induced endothelial cell network formation and proliferation was investigated. In this chapter the role of antiangiogenic factors disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1) and regulator of calcineurin-1-4 (RCAN1-4) in P CM-induced endothelial cell network

formation and proliferation was investigated. In chapter 3, ADAMTS1 expression was localised to the vasculature in endometrial adenocarcinoma. Previous studies have demonstrated that VEGF-A-induced angiogenesis is negatively regulated by secretion of a ADAMTS1 and RCAN1-4 (Iruela-Arispe et al., 2003; Minami et al., 2004). In 1997 ADAMTS1 was first identified as an inflammatory regulated gene in a murine colon adenocarcinoma cell line (Kuno et al., 1997). ADAMTS1 has been shown to inhibit VEGF-A-induced angiogenesis through different mechanisms (Iruela-Arispe et al., 2003). In one angiogenesis inhibitory mechanism ADAMTS1 can sequester VEGF-A and prevent its binding to the VEGFR2 receptor thereby inhibiting human aortic endothelial cell proliferation (Luque et al., 2003). Alternatively, ADAMTS1 inhibits angiogenesis by facilitating the cleavage of antiangiogenic thrombospondin peptides which can inhibit FGF2-driven bovine aortic endothelial cell proliferation (Lee et al., 2006). ADAMTS1 can also be upregulated by growth factors treatment, such as VEGF-A, in endothelial cells (Xu et al., 2006) and thereby acts as a negative regulator mediating the actions of VEGF-A. These studies indicate that ADAMTS1 may act as a regulator of angiogenesis in endometrial cancers.

RCAN1, also known as Down's syndrome candidate region-1, was originally identified on chromosome 21 in individuals with Down's syndrome (Fuentes et al., 2000). Individuals with Down's syndrome have an extra copy of chromosome 21 and have lower rates of many cancers. A recent study by Baek et al. has provided evidence to show that the overexpression of RCAN1 in Down's syndrome can suppress tumour growth (Baek et al., 2009). RCAN1 has 4 splice variants from exons 1, 2, 3 and 4 (Harris et al., 2005). Although exon 2 is thought to be non functional and exon 3 only contains 3 aminoacids, RCAN1-exon1 (RCAN1-1) and RCAN1-exon4 (RCAN1-4) are expressed by endothelial cells and affect endothelial cell function (Qin et al., 2006). RCAN 1-4 is an inhibitor of calcineurin and by binding to calcineurin and preventing the calcineurin-dependent dephosphorylation of NFAT, RCAN1-4 can impede NFAT-induced gene transcription (Baek et al., 2009). Recently, the RCAN1-4 isoform was identified as a negative regulator of NFAT-

induced CXCL8 in endometrial epithelial cells (Maldonado-Perez et al., 2009). RCAN1-4 isoform has previously been shown to inhibit CXCL8 expression and increase ADAMTS1 expression in VEGF-induced angiogenesis (Minami et al., 2004). In chapter 4 CXCL8 was found to regulate P CM-induced network formation and proliferation in a CXCR2 and ERK1/2 dependent manner. In addition, CXCL8 expression has also been shown to be regulated by NFAT in endothelial cells (Boss et al., 1998). Therefore, the role of RCAN1-4 in regulating P CM-induced endothelial cell network formation and proliferation via CXCL8 was investigated.

The data presented in this chapter demonstrate that antiangiogenic factors such as ADAMTS1 and regulators such as RCAN1-4 balance the proangiogenic factors mediating endothelial cell network formation and proliferation to control vascular cell function.

6.3 Results

6.3.1 Timecourse of ADAMTS1 mRNA expression in CM treated HUVECs.

In order to investigate if ADAMTS1 could be involved in regulating P CM-induced endothelial cell network formation and proliferation; HUVECs were treated with V CM or P CM for 0.5, 1, 2, 3, 4, 6, 16 and 24hrs after which ADAMTS1 expression was investigated (Fig. 67). The expression of ADAMTS1 was significantly upregulated at 1 and 4hrs after treatment with P CM compared to V CM (Fig. 67A, $P<0.05$). To confirm that ADAMTS1 is regulated by angiogenic factors such as CXCL8, HUVECs were treated with recombinant CXCL8 (10ng/ml) for 1, 3 and 4hrs after which mRNA expression of ADAMTS1 was examined (Fig. 67B). ADAMTS1 mRNA was significantly upregulated after 3hrs of treatment with recombinant CXCL8 compared to control EBM1% treatment (Fig. 67B, $P<0.05$).

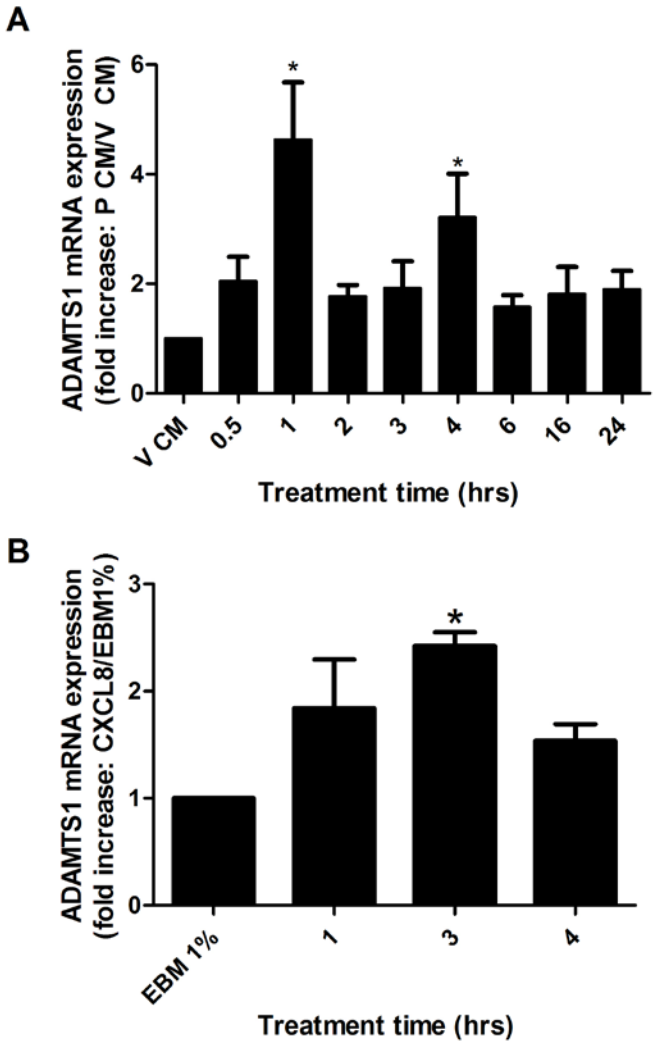


Fig. 67. ADAMTS1 mRNA expression is increased by P CM and CXCL8. A, HUVECs were treated with V CM or P CM for 0.5,1,2,3,4,6,16,24 hrs and B, HUVECs were treated with EBM1% or recombinant CXCL8 for 1, 3, 4hrs and mRNA expression of ADAMTS1 was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

6.3.2 The effect of ADAMTS1 siRNA on P CM-induced endothelial cell network formation and proliferation.

ADAMTS1 has been shown to inhibit endothelial cell proliferation and in vivo angiogenesis (Iruela-Arispe et al., 2003). Therefore, it is possible that the antiangiogenic properties of ADAMTS1 are partially responsible for regulating P CM-induced endothelial cell functions. In order to investigate this proposed antiangiogenic effect of ADAMTS1 in P CM-induced endothelial cell network formation and proliferation, ADAMTS1 siRNA, as shown in chapter 2, was used to inhibit ADAMTS1 expression in HUVECs.

HUVECs were infected with Control siRNA and ADAMTS1 siRNA for 48hrs. Thereafter, cells were incubated with V CM or P CM and network formation and proliferation assays were performed. Addition of ADAMTS1 siRNA to HUVECs significantly increased P CM-induced network formation (Fig.68A, $P<0.05$) and proliferation (Fig.68B, $P<0.05$) compared to Control siRNA cells treated with P CM. This suggests that an increase in ADAMTS1 expression levels will negatively affect endothelial cell network formation and proliferation as ADAMTS1 is a negative regulator of P CM-induced network formation and proliferation.

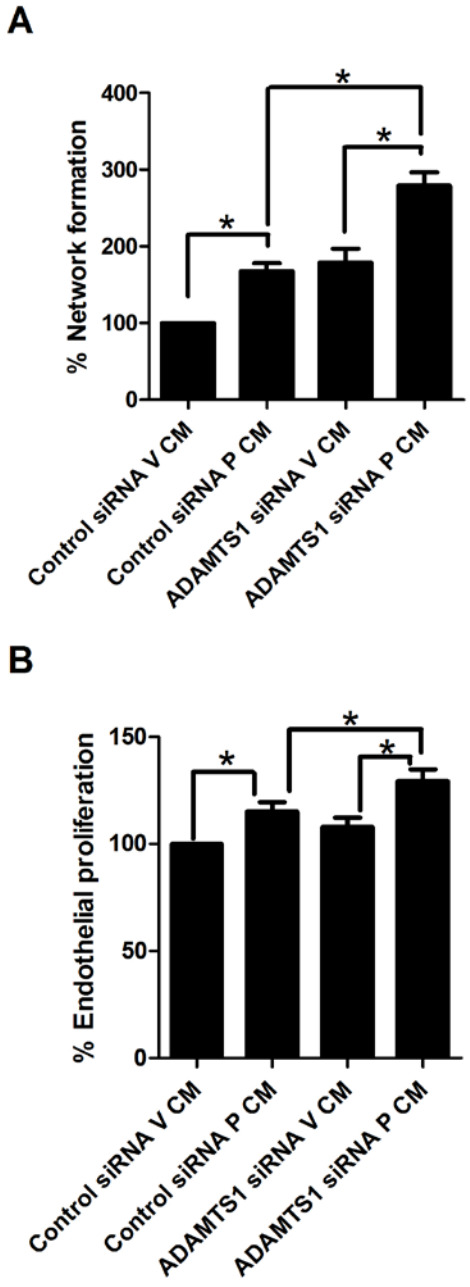


Fig.68. ADAMTS1 siRNA increases endothelial cell network formation and proliferation. HUVECs were incubated with Control siRNA or ADAMTS1 siRNA for 48hrs prior to treatment. Subsequently, Control siRNA or ADAMTS1 siRNA HUVECs were treated with V CM or P CM and network formation (A) and proliferation (B) assays were performed. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 4 independent experiments.

6.3.3 RCAN1 mRNA expression is upregulated in P CM treated HUVECs.

RCAN1-exon4 (RCAN1-4) is expressed by endothelial cells and regulates endothelial cell function (Qin et al., 2006). In order to investigate the role of RCAN1-4 in endothelial cell network formation and proliferation, HUVECs were treated with P CM for 0.5, 1, 2, 3, 4, 6, 16 and 24hrs after which RCAN1 expression was investigated (Fig.69A). The expression of RCAN1 was significantly upregulated at 3hrs after treatment with P CM compared to V CM (Fig.69A, $P<0.05$).

NFAT has been shown to upregulate RCAN1-4 expression and this can negatively regulate CXCL8 (Maldonado-Perez et al., 2009). In addition, CXCL8 can activate NFAT (Boss et al., 1998) therefore, RCAN1-4 could itself be upregulated by CXCL8. As shown in chapter 4, CXCL8, in the P CM, activates MEK-ERK1/2 via the CXCR2 receptor which increases the endothelial expression of CXCL8. To examine if this signal transduction pathway is involved in P CM induction of RCAN1 by CXCL8, HUVECs were treated with P CM in the absence or presence of inhibitors of CXCR2 (SB225002) and ERK1/2 (PD98059) (Fig.69B). After 3hrs of treatment, the expression of RCAN1 was analysed (Fig.69B). The CXCR2 inhibitor, SB225002, significantly reduced RCAN1 expression (Fig.69B, $P<0.05$) however, the inhibitor of ERK1/2, PD98059, had no effect on RCAN1 expression (Fig.69B). This indicates that the signal transduction pathway induced by CXCL8 and regulating RCAN1 expression are different to those regulating the production of P CM-induced proangiogenic growth factors, as seen in chapter 4.

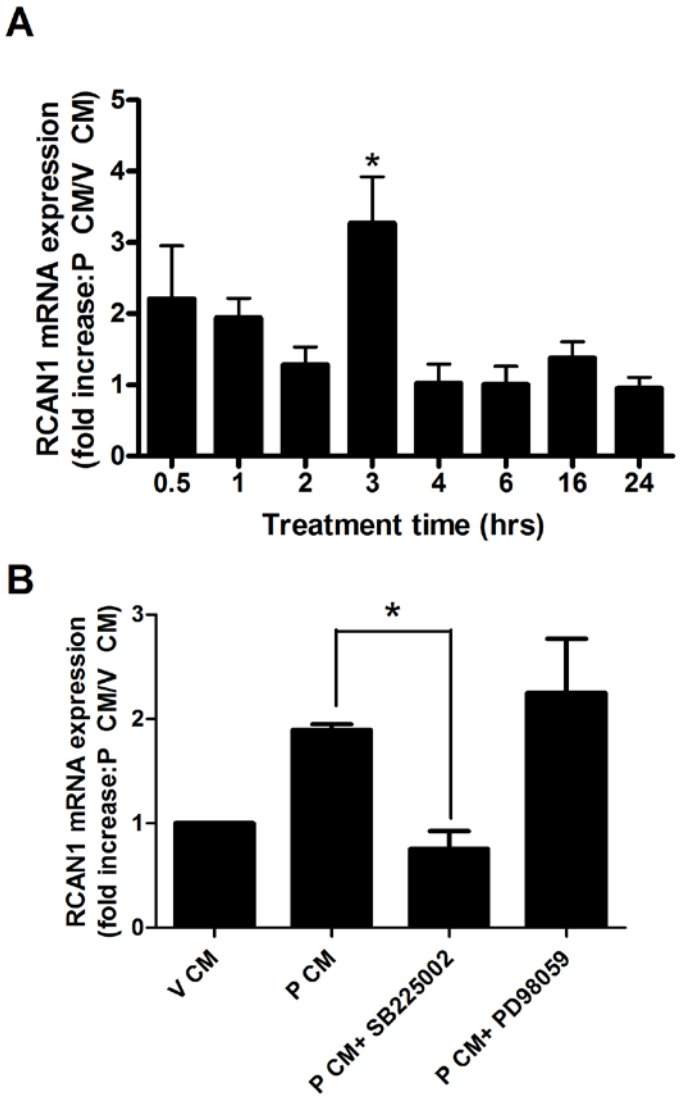


Fig.69. RCAN1 mRNA expression after P CM treatment. A, HUVECs were treated with V CM or P CM for 0.5,1,2,3,4,6,16,24 hrs and B, HUVECs were treated for 3hrs with V CM or P CM in the absence/presence of SB225002 (CXCR2 antagonist) or PD98059 (ERK1/2 inhibitor) and mRNA expression of RCAN1 was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 5 independent experiments.

6.3.4 Overexpression of RCAN1-4 negatively regulates endothelial cell network formation and proliferation.

As RCAN1-4 overexpression can inhibit angiogenesis (Minami et al., 2004), and it is upregulated in HUVECs treated with P CM, its role in HUVEC network formation and proliferation was investigated using an adenoviral construct to overexpress RCAN1-4. As shown in section 2.9.3.3, the addition of 100MOI of adenovirus RCAN1-4 (Ad-RCAN1) increased the expression of RCAN1 more than 100 fold above the control. Control adenovirus (Ad-Control) did not alter RCAN1 basal levels. Therefore, HUVECs were infected with 100MOI of an adenovirus control or adenovirus RCAN1 and subsequently treated with V CM or P CM to examine endothelial cell network formation and proliferation. In the Ad-Control treated HUVECs, P CM significantly increased endothelial cell network formation (Fig. 70A, $P<0.05$) and proliferation (Fig. 70B, $P<0.05$). However, in HUVECs infected with Ad-RCAN1 to overexpress RCAN1-4, the P CM-induced increase in network formation (Fig. 70A, $P<0.05$) and proliferation (Fig. 70B, $P<0.05$) was inhibited compared to Ad-Control cells treated with P CM. This suggests that an increase in the levels of RCAN1-4 negatively regulates endothelial cell network formation and proliferation.

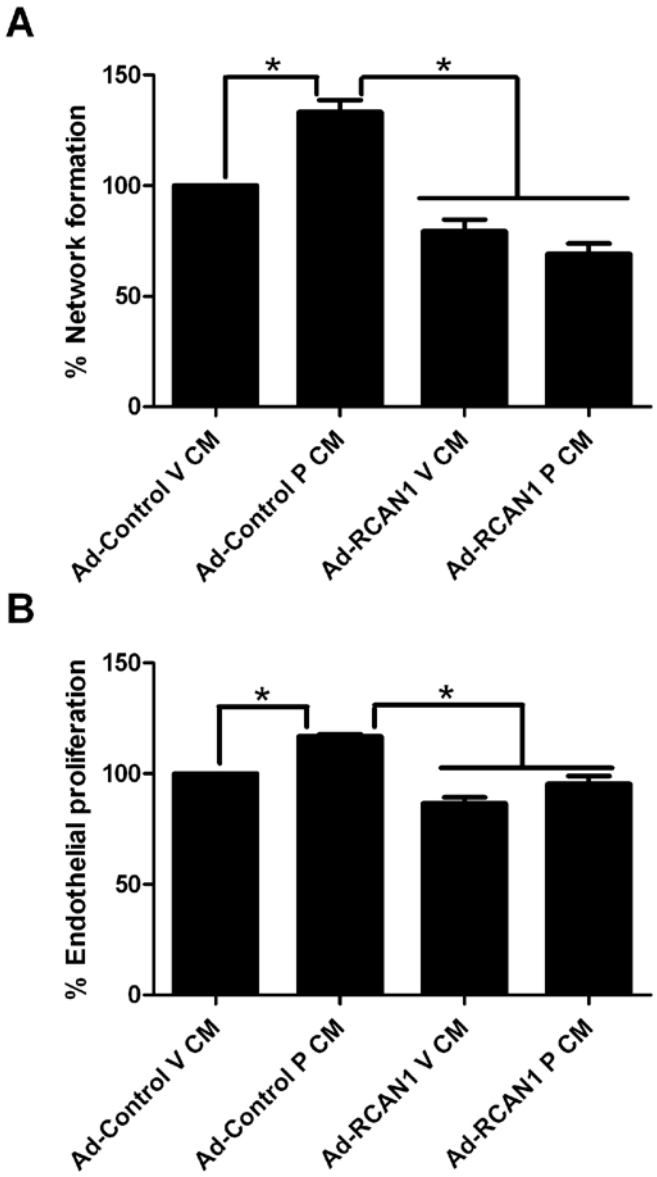


Fig. 70. The effect of RCAN1-4 overexpression on endothelial cell network formation and proliferation. HUVECs were transfected with 100MOI of adenovirus construct containing non-target (Ad-Control) or RCAN1-4 cDNA (Ad-RCAN1). Subsequently, cells were treated with V CM or P CM and network formation (A) and proliferation (B) were assessed. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of 4 independent experiments.

6.3.5 The effect of RCAN1-4 overexpression on CXCL8 mRNA expression in endothelial cells.

In chapter 4, CXCL8 in the P CM was found to regulate endothelial cell network formation and proliferation via the CXCR2 receptor. In addition, CXCL8 mRNA expression was upregulated in a CXCR2 dependent manner suggesting that CXCL8 acting via the CXCR2 receptor regulates a positive feedback loop promoting CXCL8 expression and endothelial cell function. RCAN1-4 has previously been shown to negatively regulate CXCL8 expression (Maldonado-Perez et al., 2009). Therefore, to determine if RCAN1-4 overexpression was preventing endothelial cell network formation and proliferation via the inhibition of CXCL8 expression, HUVECs were infected with Ad-Control and Ad-RCAN1 adenovirus and treated with V CM or P CM after which CXCL8 mRNA expression was examined (Fig. 71). CXCL8 mRNA expression in Ad-Control cells was significantly elevated after 3hrs of P CM treatment compared to V CM treatment (Fig. 71A, $P<0.05$). In Ad-RCAN1 cells, this increase in CXCL8 mRNA induced by P CM was inhibited compared to Ad-Control cells treated with P CM (Fig. 71A, $P<0.05$). To confirm the possibility that RCAN1-4 overexpression could negatively regulate CXCL8-induced expression of endothelial CXCL8, adenovirus infected HUVECs were treated with recombinant CXCL8 or control media (EBM1%) and CXCL8 expression was examined. In Ad-Control cells the addition of CXCL8 recombinant protein significantly increased CXCL8 expression, compared to Ad-Control cells treated with EBM1% (Fig. 71B, $P<0.05$). In Ad-RCAN1 cells, the induction of CXCL8 expression by recombinant CXCL8 was inhibited (Fig. 71B, $P<0.05$).

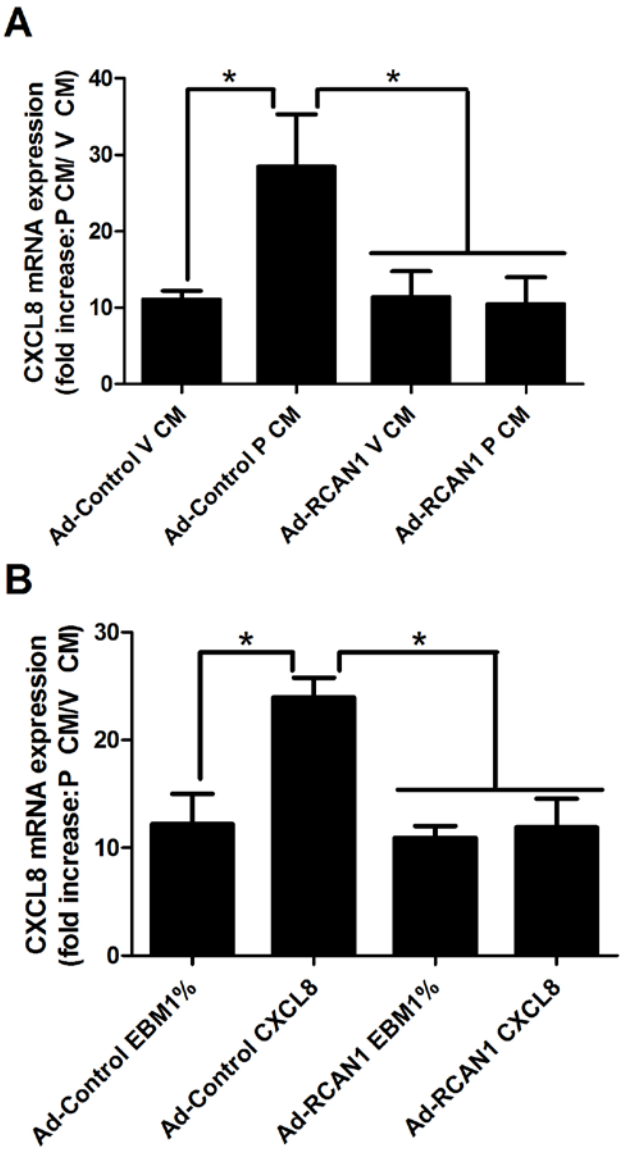


Fig. 71. CXCL8 mRNA expression is decreased by RCAN1-4 overexpression at 3hrs. HUVECs were transfected with 100MOI of adenovirus construct containing non-target (Ad-Control) or RCAN1 cDNA (Ad-RCAN1). Subsequently, cells were treated with V CM or P CM (A) or EBM1% and CXCL8 (B) and CXCL8 expression was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of 5 independent experiments.

Next, the possibility that addition of recombinant CXCL8 to RCAN1-4 overexpressing HUVECs could rescue P CM-induced endothelial cell network formation and proliferation was investigated (Fig 72).

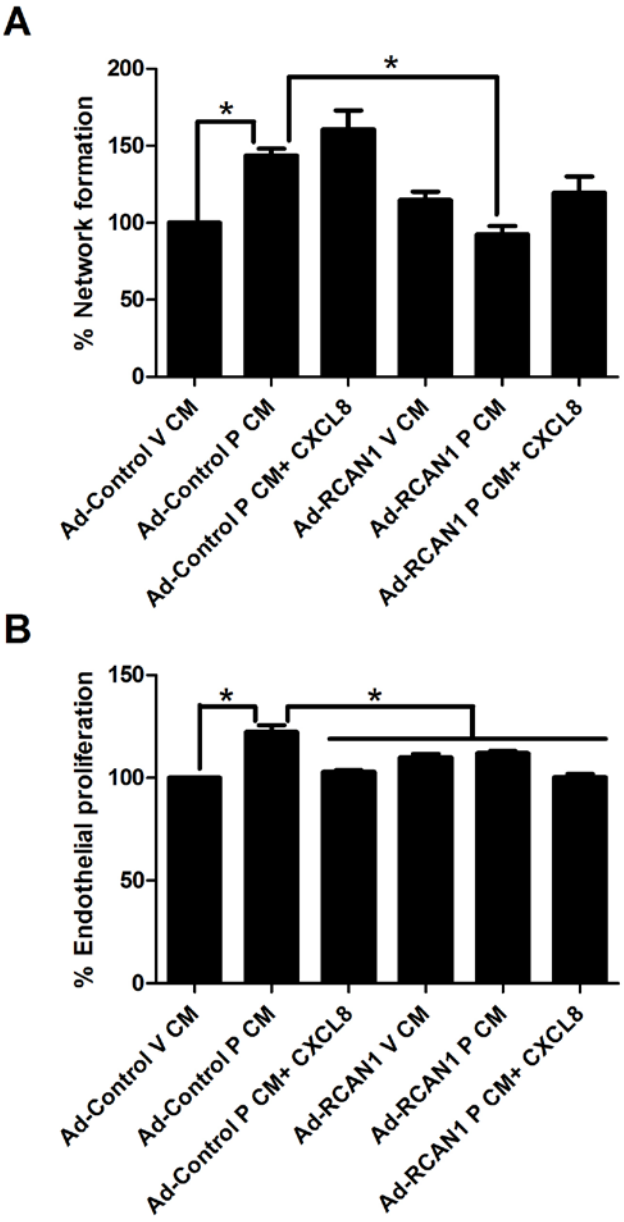


Fig. 72. Addition of CXCL8 to RCAN1-4 overexpressing cells does not rescue network formation or proliferation. HUVECs were transfected with 100MOI of adenovirus construct containing non-target (Ad-Control) or RCAN1-4 cDNA (Ad-RCAN1). Subsequently, cells were treated with V CM, P CM or P CM plus recombinant CXCL8 (10ng/ml) and network formation (A) and proliferation (B) were assessed. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM of at least 4 independent experiments.

Ad-Control and Ad-RCAN1 HUVECs were treated with V CM and P CM with or without recombinant CXCL8 (Fig. 72). There was no significant difference in HUVEC network formation (Fig. 72A) or proliferation (Fig. 72B) seen with Ad-RCAN1 cells treated with P CM and CXCL8 compared to Ad-RCAN1 cells treated with P CM alone. This demonstrates that the addition of recombinant CXCL8 did not rescue the RCAN1-4 overexpressing cells. Therefore, it is possible that RCAN1-4 is regulating the expression of other angiogenic proteins which may mediate P CM-induced endothelial cell proliferation.

The overexpression of RCAN1-4 in HUVECs has been shown to decrease angiogenic protein expression but it may also increase the expression of antiangiogenic proteins. For example, data by Minami et al. found that RCAN1-4 overexpression in HUVECs increases ADAMTS1 expression (Minami et al., 2004). As ADAMTS1 expression is increased after P CM treatment (Fig. 67) and ADAMTS1 expression inhibits P CM-induced endothelial cell network formation and proliferation (Fig.68), it is possible that RCAN1-4 may regulate endothelial cell function via ADAMTS1 expression. Hence, to investigate the role of RCAN1-4 in the regulation of ADAMTS1, Ad-Control and Ad-RCAN1 HUVECs were treated with V CM or P CM and ADAMTS1 mRNA expression was examined (Fig. 73A). ADAMTS1 mRNA expression was significantly increased in Ad-RCAN1 HUVECs treated with P CM compared to control HUVECs treated with P CM (Fig. 73, $P<0.05$). This indicates that RCAN1-4 is augmenting P CM upregulation of ADAMTS1. Recombinant CXCL8 treatment was found to increase ADAMTS1 expression (Fig. 67B) therefore, the effect of RCAN1-4 overexpression on ADAMTS1 expression induced by recombinant CXCL8 was examined (Fig. 73B). In Ad-RCAN1 HUVECs treated with recombinant CXCL8 had elevated levels of ADAMTS1 mRNA but this was not significantly different to Ad-Control cells treated with recombinant CXCL8 (Fig. 73B).

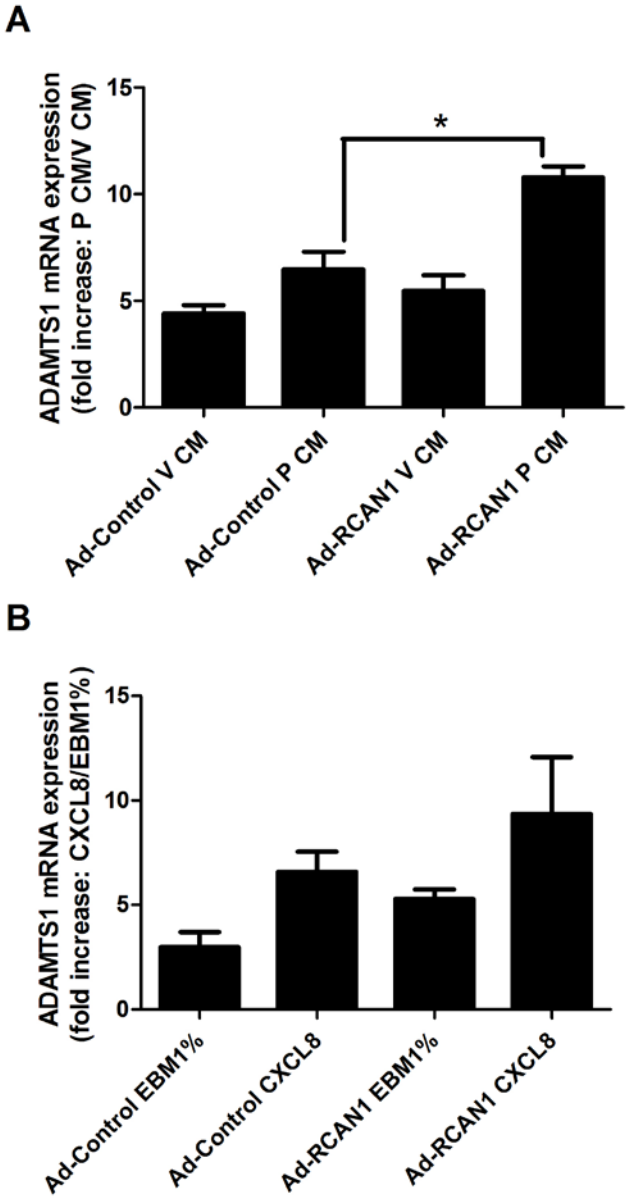


Fig. 73. ADAMTS1 expression increased by RCAN1 overexpression. HUVECs were transfected with 100MOI adenovirus control (Ad-Control) or adenovirus RCAN1-4 (Ad-RCAN1) for 24hrs prior treatment. Ad-HUVECs were treated with for 4hrs with V CM or P CM (A) or for 3hrs with EBM1% and recombinant CXCL8 (10ng/ml) (B). After treatment, mRNA expression of ADAMTS1 was determined by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 4 independent experiments.

6.3.6 Decreasing endothelial RCAN1-4 expression differentially regulates P CM-induced endothelial cell network formation and proliferation.

Next the effect of reducing endothelial RCAN1 expression, with use of a lentiviral construct containing RCAN1-4 short hairpin RNA (shRNA), on P CM-induced endothelial cell network formation and proliferation, was investigated. As increasing RCAN1-4 expression in HUVECs decreased endothelial cell network formation and proliferation (Fig. 70), decreasing RCAN1-4 could possibly increase endothelial cell network formation and proliferation. Therefore, HUVECs were infected with a non-target control lentivirus (lv-Control) or lentivirus containing RCAN1-4 shRNA (lv-RCAN1). As shown in section 2.9.3.4, the expression of RCAN1 in lv-RCAN1-4 cells was significantly decreased compared to lv-Control cells. To examine the effect of decreased RCAN1-4 expression on endothelial cell network formation and proliferation, lv-Control and lv-RCAN1 HUVECs were treated with V CM and P CM (Fig. 74). The lv-Control cells exhibited normal response to P CM because P CM treatment significantly increased endothelial cell network formation (Fig. 74A, $P<0.05$) and proliferation (Fig. 74B, $P<0.05$) compared to V CM treatment. In lv-RCAN1 HUVECs the addition of P CM significantly increased endothelial cell network formation above the levels seen in lv-Control cells treated with P CM (Fig. 74A, $P<0.05$). However, contrary to what was observed for the network formation, inhibition of RCAN1 expression with shRNA did not elevate cellular proliferation (Fig. 74B). lv-RCAN1 HUVECs treated with P CM exhibited a decrease in proliferation compared to lv-Control P CM (Fig. 74B, $P<0.05$). There was also a significant decrease in the proliferation of lv-RCAN1 HUVECs treated with V CM compared to lv-Control HUVECs treated with V CM (Fig. 74B, $P<0.05$). This was an unexpected finding because HUVEC proliferation was proposed to increase in lv-RCAN1 cells.

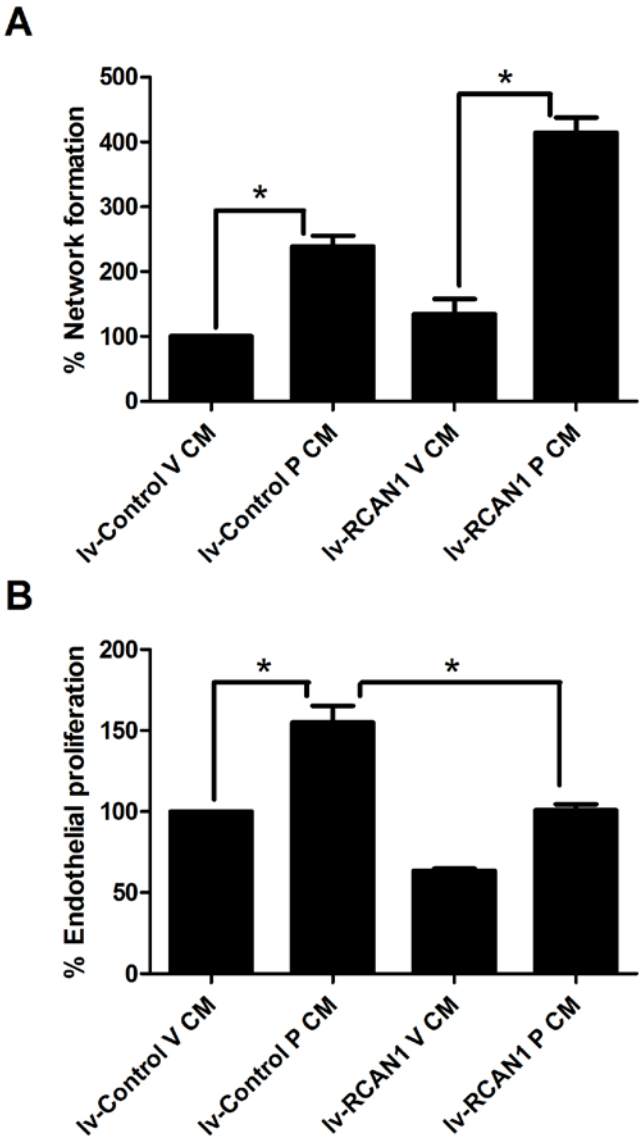


Fig. 74. The effect of decreasing RCAN1 expression on endothelial network formation and proliferation. HUVECs were transfected with 5MOI of lentiviral control (lv-Control) or lentivirus RCAN1 (lv-RCAN1) for 24hrs and allowed to recover for 12hrs prior to experiments. Lentivirus HUVECs were treated with V CM or P CM and network formation (A) and proliferation assays (B) were performed. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 4 independent experiments.

6.3.7 The effect of RCAN1-4 shRNA on P CM induced mRNA expression of COX-2, CXCL8 and ADAMTS1.

As mentioned previously, RCAN1-4 is a negative regulator of NFAT gene transcription (Baek et al., 2009). In endothelial cells, one angiogenic target of NFAT is cyclooxygenase 2 (Hernandez et al., 2001). As shown in chapter 4, the increase in angiogenic gene expression of FGF2, CXCL1 and CXCL8 was partially dependent on COX-2 expression. In addition, data in chapter 5 show that the COX-2 product, $PGF_{2\alpha}$ promotes endothelial cell network formation. Therefore, the possibility was investigated that the elevation in network formation, seen after a reduction in RCAN1-4 expression, was due to an enhancement of COX-2 expression. The expression of COX-2 was examined in lv-Control and lv-RCAN1 HUVECs treated for 3hrs with V CM, P CM, EBM1% or CXCL8 (Fig.75). In lv-RCAN1 cells treated with P CM, there was a significant increase in COX-2 mRNA expression compared to lv-Control cells treated with V CM but not P CM (Fig.75, $P<0.05$).

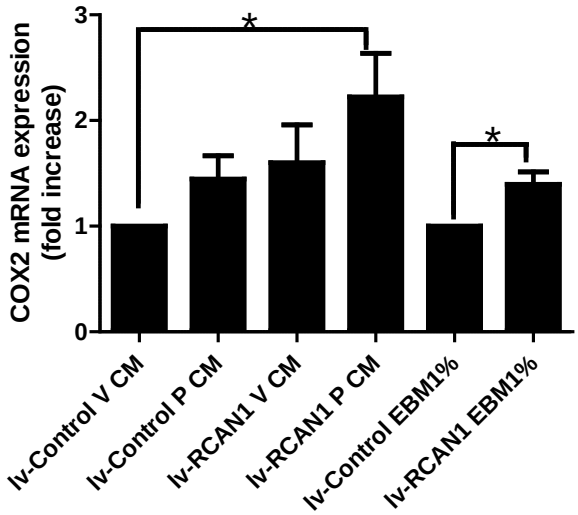


Fig.75. The effect of lv-RCAN1 on COX-2 mRNA expression in HUVECs. HUVECs were transfected with 5MOI of lentiviral control (lv-Control) or lentivirus RCAN1 (lv-RCAN1) for 24hrs and allowed to recover for 12hrs prior to experiments. lv-Control and lv-RCAN1 HUVECs were treated with V CM, P CM, EBM1% or CXCL8 for 3hrs after which COX-2 mRNA expression was analysed by quantitative RT-PCR. (* represents statistical significance; $P<0.05$). Data are represented as mean ± SEM from 4 independent experiments.

COX-2 expression in lv-Control cells treated with P CM was not significantly different to lv-Control cells treated with V CM (Fig.75) although as shown in chapter 4, P CM treatment does significantly increase COX-2 mRNA levels in untransfected cells. The level of COX-2 mRNA expression was also significantly increased in untreated lv-RCAN1 cells (lv-RCAN1 EBM1%) compared to untreated lv-Control cells (lv-Control EBM1%) (Fig.75, $P<0.05$) further suggesting that COX-2 mRNA expression is increased when RCAN1-4 expression is decreased.

Earlier in this chapter, it was shown that CXCL8 mRNA expression was decreased by RCAN1-4 overexpression suggesting that RCAN1-4 negatively regulates endothelial CXCL8 expression. In order to investigate whether decreasing RCAN1 expression would increase CXCL8 expression, HUVECs were infected with lv-Control and lv-RCAN1 and treated for 1, 3 and 4 hrs with V CM and P CM after which the expression of CXCL8 was examined (Fig.76A). Unexpectedly, the expression of CXCL8 was significantly decreased in lv-RCAN1 cells treated with P CM compared to lv-Control cells treated with P CM after 1 and 4 hrs of treatment (Fig.76A, $P<0.05$). This indicates that contrary to the expected increase in NFATc1-regulated gene expression, due lack of RCAN1-4 negative feedback, CXCL8 expression was not increased. To confirm the role of calcineurin-NFAT signalling in CXCL8 mRNA expression, lv-Control and lv-RCAN1 cells were coincubated with P CM and the calcineurin inhibitor cyclosporin A (CsA) (Fig.76A). The expression of CXCL8 in lv-RCAN1 cells treated with P CM was significantly decreased by the addition of CsA (Fig.76A, $P<0.05$) after 1 hr of treatment, compared to P CM alone. This suggests that even when gene transcription is decreased, calcineurin-NFAT activation is still regulating gene transcription of CXCL8 as cyclosporin A can inhibit these low levels of endothelial CXCL8 expression.

Previously in this chapter, it was shown that ADAMTS1 mRNA expression was increased by RCAN1-4 overexpression (Fig. 73). Conversely, it may be expected that decreasing RCAN1-4 expression would decrease ADAMTS1 expression. Therefore, the expression of ADAMTS1 in lv-Control and lv-RCAN1 cells was

examined. Contrary to expectations, the expression of ADAMST1 was significantly increased in lv-RCAN1 cells treated with P CM compared to lv-Control cells treated with P CM (Fig.76B). To investigate the role of calcineurin-NFAT transcription in ADAMTS1 expression, cells were co-treated with P CM and cyclosporin A (Fig.76B). Cyclosporin A treatment did not decrease ADAMTS1 mRNA expression induced by P CM (Fig.76B). These data suggest that an increase or decrease in NFAT gene transcription alone may not affect ADAMTS1 expression.

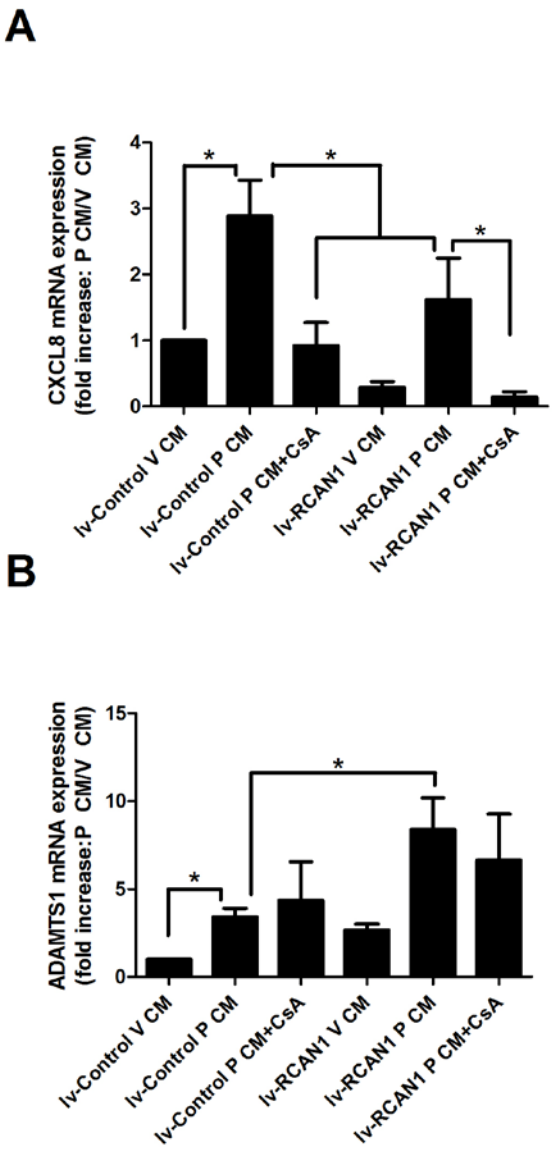


Fig.76. The effects of lv-RCAN1 on CXCL8 and ADAMTS1 mRNA expression. HUVECs were transfected with 5MOI of lentiviral control (lv-Control) or lentivirus RCAN1 (lv-RCAN1) for 24hrs prior to experiments. Subsequently, lv-Control and lv-RCAN1 HUVECs were treated for 1, 3 and 4 hrs with V CM, P CM or P CM plus cyclosporin A (P CM+ CsA). After

which, CXCL8 (A) and ADAMTS1 (B) expression was analysed by quantitative RT-PCR. (* represents statistical significance; P<0.05). Data are represented as mean ± SEM from at least 3 independent experiments.

6.4 Discussion

Angiogenesis is regulated by a balance between pro- and antiangiogenic factors. Data in chapters 4 and 5, of this thesis have established a proangiogenic role for FGF2, CXCL1 and CXCL8 in P CM-induced endothelial cell network formation and proliferation. In this chapter, the role of antiangiogenic ADAMTS1 and RCAN1-4 in P CM-induced endothelial cell network formation and proliferation was investigated. Antiangiogenic ADAMTS1 has been shown to inhibit VEGF-A induced angiogenesis through a variety of mechanisms (Iruela-Arispe et al., 2003). In one angiogenesis inhibitory mechanism, ADAMTS1 can sequester VEGF-A and prevent its binding to the VEGFR2 receptor thereby inhibiting human aortic endothelial cell proliferation (Luque et al., 2003). Alternatively, ADAMTS1 inhibits angiogenesis by facilitating the cleavage of antiangiogenic thrombospondin peptides which can inhibit FGF2-driven bovine aortic endothelial cell proliferation (Lee et al., 2006). ADAMTS1 can also be upregulated by growth factor treatment, such as VEGF-A, in endothelial cells (Xu et al., 2006). In this chapter, the expression of ADAMTS1 in HUVECs by P CM treatment was investigated and ADAMTS1 was found to be elevated after 1hr and 4hrs of P CM treatment compared to V CM. This elevation of ADAMTS1 was in a reciprocal manner to the expression observed for proangiogenic growth factors CXCL1 and CXCL8 which were maximally induced at 3hrs (see section 4.4.2). These findings indicate that angiogenic factors in the P CM may regulate ADAMTS1 expression. To confirm this, recombinant CXCL8 was used and found to stimulate endothelial ADAMTS1 expression after 3hrs of treatment, suggesting that CXCL8 in the P CM could induce ADAMTS1 expression.

To verify the antiangiogenic role that ADAMTS1 may play in P CM-induced endothelial cell network formation and proliferation, ADAMTS1 siRNA was used to inhibit HUVEC ADAMTS1 expression. Inhibition of ADAMTS1 expression in HUVECs increased P CM-induced endothelial cell network formation and proliferation. This is in agreement with data from Iruela-Arispe et al. who

demonstrated that ADAMTS1 inhibits bovine aortic endothelial cell proliferation and angiogenesis in vivo (Iruela-Arispe et al., 2003; Luque et al., 2003). These data indicate that ADAMTS1 negatively regulates endothelial cell network formation and proliferation induced by P CM.

RCAN1 is also known as Down syndrome critical region 1 (DSCR1), Adapt78 and MCIP1 (Davies et al., 2007). RCAN1 has 4 splice variants from exons 1, 2, 3 and 4 (Harris et al., 2005). Although exon 2 is thought to be non functional and exon 3 only contains 3 amino acids, RCAN1-exon1 (RCAN1-1) and RCAN1-exon4 (RCAN1-4) are expressed by endothelial cells and regulate endothelial cell function (Qin et al., 2006). RCAN 1-4 binding to calcineurin prevents the calcineurin-dependent dephosphorylation of NFAT hence, RCAN1-4 can impede NFAT-induced gene transcription (Baek et al., 2009). A study by Minami et al. showed that RCAN1-4 overexpression can inhibit NFAT-induced VEGF-A expression in HUVECs and thereby inhibit VEGF-A-induced HUVEC network formation and proliferation (Minami et al., 2004). Further in vivo studies using a matrigel plug assay and melanoma xenografts indicate that VEGF-A-induced angiogenesis is inhibited by RCAN1-4 overexpression (Minami et al., 2004). This suggests that overexpression of RCAN1-4 has antiangiogenic actions. These antiangiogenic actions are further supported by the observation that individuals with Down's syndrome, who have an extra copy of the RCAN1 gene locus, display reduced incidences of solid tumours and reduced tumour growth rates (Baek et al., 2009).

To investigate the possibility that RCAN1 may be regulating endothelial cell network formation and proliferation, the expression of RCAN1 in HUVECs was determined by quantitative RT-PCR. RCAN1 was upregulated after 3hrs of treatment with P CM compared to V CM. This suggested that growth factors in the P CM are increasing RCAN1 expression. Using the CXCR2 antagonist together with P CM decreased HUVEC expression of RCAN1, indicating that CXCL8 signals through the CXCR2 receptor to upregulate RCAN1. In contrast to the expression of growth factors which are upregulated via the ERK1/2 pathway, RCAN1 expression was

found to independent of ERK1/2 activation. This is in agreement with data by Iizuka et al. who found that RCAN1 upregulation by VEGF-A treatment was independent of ERK1/2 activation (Iizuka et al., 2004). Similarly, treatment of HUVECs with PLC, PI3K, P38 and JNK inhibitors had no effect on the expression of RCAN1 induced by P CM (data not included). In addition, Yao et al. showed using HUVECs that VEGF-A-induced RCAN1 expression was independent of ERK1/2, P38 and PI3K (Yao and Duh, 2004). Iizuka et al. also found that in HUVECs, RCAN1 upregulation by VEGF-A was independent of PI3K and P38 but dependent on calcium-calcineurin signalling (Iizuka et al., 2004). It is important to note that the cytokine receptors such as CXCR2, a GPCR, are not assumed to activate NFAT unless they can stimulate calcium pathways leading to calcineurin (Rao et al., 1997). However, the CXCR2 receptor can transactivate the tyrosine kinase receptor VEGFR2 in human microvascular endothelial cells (Martin et al., 2009) (Petreaca et al., 2007) which previous research has shown can activate endothelial cell calcineurin-NFAT signalling (Schweighofer et al., 2007). Therefore, it is possible that CXCL8, in the P CM, signalling through the CXCR2 receptor on endothelial cells could regulate NFAT activation via a mechanism involving the transactivation of tyrosine kinase receptors such as VEGFR2.

Next to examine the effect that increasing RCAN1-4 expression would have on P CM-induced endothelial cell network formation and proliferation, an adenovirus RCAN1-4 construct was used. As the RCAN1-4 isoform has previously been shown to inhibit CXCL8 expression (Maldonado-Perez et al., 2009) and angiogenesis (Minami et al., 2004), this was the isoform investigated. Data herein demonstrates that P CM-induced endothelial cell network formation and proliferation was reduced in HUVECs infected with RCAN1-4 adenovirus. This is in line with previous data showing that RCAN1-4 overexpression inhibits VEGF-induced endothelial cell network formation and proliferation (Minami et al., 2004).

The mechanism by which RCAN1-4 regulated endothelial cell network formation and proliferation was further investigated. Ligand binding to membrane receptors

such as GPCRs can elevate intracellular calcium which activates calcineurin (Rao et al., 1997). Activated calcineurin dephosphorylates cytoplasmic NFAT allowing its translocation to the nucleus where it stimulates gene transcription (Rao et al., 1997). RCAN1-4 can inhibit NFAT induced transcription because RCAN1-4 and NFAT compete for binding to calcineurin (Martinez-Martinez et al., 2009). This would imply that with more RCAN1-4 there would be more competition for calcineurin, which may decrease NFAT-induced gene transcription. CXCL8 has been identified as a target of NFAT gene transcription negatively regulated by RCAN1-4 (Maldonado-Perez et al., 2009). In chapter 4, CXCL8 activation of the CXCR2 receptor was shown to regulate CXCL8 expression and endothelial cell network formation and proliferation suggesting that CXCL8 is regulated by a positive feedback loop. Therefore, the possibility was investigated that the inhibitory effect of RCAN1-4 on endothelial cell network formation and proliferation was due to a decrease in NFAT-dependent endothelial CXCL8 expression. Subsequent in vitro investigations found that in HUVECs, RCAN1-4 overexpression with adenovirus inhibited P CM-induced CXCL8 expression. Similarly, HUVEC CXCL8 expression induced by recombinant CXCL8 treatment was also inhibited by RCAN1-4 overexpression. In agreement, a previous report by Minami et al. showed that CXCL8 expression is downregulated in HUVECs overexpressing RCAN1-4 (Minami et al., 2004). This indicates that the CXCL8 autocrine loop enhancing endothelial cell network formation and proliferation is regulated by RCAN1-4. However, addition of recombinant CXCL8 to RCAN1-4 overexpressing cells did not counteract the effects of RCAN1-4 overexpression as it did not rescue P CM-induced endothelial cell network formation and proliferation. This indicates that either RCAN1-4's inhibitory effect on endothelial cell function is not regulated solely by CXCL8, or the concentration of CXCL8 was not sufficient to overcome the action of RCAN1-4 overexpression in HUVECs. Previous studies have shown that RCAN1 can regulate the expression of eNOS in HUVECs (Riper et al., 2008). Additionally, Hesser et al. showed that RCAN1-4 overexpression in HUVECs decreases inflammatory modulators such as COX-2, TF, E-selectin and VCAM-1 (Hesser et al., 2004).

Interestingly, as well as down-regulating angiogenic molecules, RCAN1-4 can up-regulate antiangiogenic molecules (Minami et al., 2004). In RCAN1-4 overexpressing cells treated with P CM, ADAMTS1 expression was significantly upregulated compared to control cells treated with P CM. This is in agreement with data from Minami et al. who showed that ADAMTS1 expression is increased in RCAN1-4 overexpressing HUVECs treated with VEGF-A or thrombin (Minami et al., 2004). These data suggest that ADAMTS1 expression is either negatively regulated by calcineurin-NFAT signalling or RCAN1-4 promotes ADAMTS1 expression via an alternative pathway.

Furthermore, it could be proposed that the induction of ADAMTS1 by RCAN1-4 together with its inhibition of CXCL8 expression maintains the balance of endothelial cell function by preventing excessive endothelial network formation and proliferation in the presence of proangiogenic growth factors (see Fig. 77).

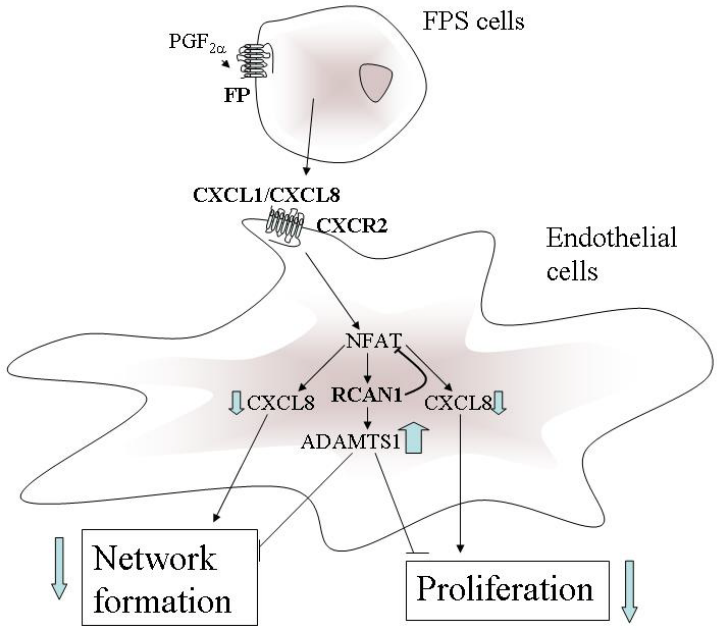


Fig. 77. Possible mechanism regulating endothelial cell network formation and proliferation in RCAN1-4 overexpressing cells. RCAN1-4 overexpression inhibits endothelial cell CXCL8 expression and increases endothelial ADAMTS1 expression resulting in a decrease in endothelial cell network formation and proliferation.

Since the overexpression of RCAN1-4 decreased endothelial cell network formation and proliferation, it was expected that decreasing the levels of RCAN1-4 expression, by lentiviral introduction of short hairpin RCAN1-4 to decrease endogenous RCAN1-4 in endothelial cells, would increase P CM-induced endothelial cell network formation and proliferation. Decreasing RCAN1-4 expression did increase P CM-induced endothelial cell network formation. However, contrary to expectations, P CM-induced endothelial cell proliferation was decreased with decreased RCAN1-4 expression. One explanation for this is that the calcineurin-NFAT pathway inhibited by RCAN1-4 is involved in cellular proliferation and the regulation of apoptosis. Hyperactivation of calcineurin can activate transcription dependent and transcription independent apoptotic pathways (Ryeom et al., 2008). For example, a transcription dependent mechanism regulated by calcineurin involves the activation of NFAT. NFAT family members are activated when dephosphorylated by calcineurin (Rao et al., 1997). This allows NFAT to translocate to the nucleus where it regulates gene transcription (Rao et al., 1997). An excess of calcineurin results in an excess of dephosphorylated NFAT which translocates to the nucleus and activates the apoptotic pathways via Fas-ligand genes (Latinis et al., 1997). The transcription independent pathway regulated by calcineurin involves the dephosphorylation of pro-apoptotic protein BAD. Both these calcineurin pathways can be regulated by the inhibition of calcineurin either by RCAN1 or a small chemical inhibitor, cyclosporin A. Therefore, the addition of cyclosporin A (CsA) to endothelial cells undergoing proliferation in normal circumstances will result in a decrease in proliferation but in an environment where calcineurin is hyperactivated, when used at the correct concentration, could result in an increase in proliferation (Ryeom et al., 2008). Hyperactivation of calcineurin can cause excessive NFAT nuclear transcription and activation of apoptotic genes. Alternatively, hyperactivated calcineurin can cause transcription independent apoptosis via dephosphorylation of apoptotic protein BAD. A recent study by Ryeom et al. investigated the effects of RCAN1-4 deficiency in tumorigenesis and found that in vivo tumour growth and angiogenesis was suppressed in RCAN null mice (Ryeom et al., 2008). Their premise was that endothelial cell proliferation was inhibited by the loss of RCAN1-4 due to

hyperactivation of calcineurin leading to apoptotic pathways (Ryeom et al., 2008). Ryeom et al. showed that VEGF-A stimulated proliferation in RCAN1-4 null endothelial cells could be restored by the addition of 10nM low dose of calcineurin inhibitor cyclosporin A (CsA) (Ryeom et al., 2008).

Although decreasing RCAN1-4 expression inhibited endothelial cell proliferation, it had the opposite effect and increased endothelial cell network formation. This suggests that as seen in chapter 4, network formation and proliferation are regulated by separate pathways induced by growth factors. Thus, the expression of angiogenic factors was investigated by QPCR. COX-2 expression was upregulated in RCAN1-4 deficient endothelial cells. In support of this, RCAN1-null mouse endothelial cells exhibited an increase in COX-2 protein levels after VEGF-A treatment (Ryeom et al., 2008). Additionally, data by Yao et al. showed in HUVECs overexpressing RCAN1-4, treated with VEGF-A, that NFATc1 activation increases COX-2 expression (Yao and Duh, 2004). This suggests that COX-2 expression can be regulated by calcineurin-NFAT and RCAN1-4 levels could affect COX-2 expression. In line with this, data by Loster et al. show that NFAT is responsible for COX-2 upregulation and secretion of prostaglandins by HUVECs (Lötzer et al., 2007). The network formation in chapter 5 was found to involve $PGF_{2\alpha}$ signalling and the production of $PGF_{2\alpha}$ was dependent on COX-2. This indicates that in RCAN1-4 deficient cells, if there is an increase in COX-2 expression, the production of $PGF_{2\alpha}$ could also be upregulated and may be responsible for the increase in network formation seen in HUVECs infected with lentivirus shRNA to decrease RCAN1-4 expression.

Interestingly, CXCL8 expression was not upregulated in RCAN1-4 deficient cells despite the evident increase in COX-2 expression and the presumed increase in NFAT activity. The reasons for this contradiction are unclear but it indicates that endothelial CXCL8 expression is not essential for autocrine signalling to enhance network formation as the lack of CXCL8 upregulation does not inhibit network formation. Addition of the calcineurin inhibitor CsA to the RCAN1-4 deficient cells

further decreased CXCL8 expression indicating that calcineurin-NFAT signalling is still needed for low levels of CXCL8 expression.

The expression of ADAMTS1 is further upregulated in RCAN1-4 deficient cells and this increase in ADAMTS1 expression was not prevented by calcineurin inhibition with CsA, in fact a trend towards an increase in ADAMTS1 expression was observed. This indicates that RCAN1-4 may play an additional role in the regulation of ADAMTS1 independently of calcineurin inhibition rather than suggesting that calcineurin may negatively regulate ADAMTS1 expression. This increase in ADAMTS1 did not prevent the increase in network formation seen in HUVECs, infected with lentiviral shRNA to deplete endogenous RCAN1-4, indicating that although a lack of ADAMTS1 enhances network formation, the inhibitory effect of an increase in ADAMTS1 on network formation must be counteracted by growth factors in the P CM. This is possible because data in chapter 4 showed that in untransfected HUVECs, P CM treatment increased network formation even though endothelial ADAMTS1 expression was upregulated.

It is evident that the mechanisms regulating endothelial cell network formation differ from those regulating endothelial cell proliferation. As seen in chapter 5, P CM-induced endothelial cell network formation was regulated by prostaglandin $F_{2\alpha}$ secretion whereas proliferation was not. In this chapter, decreasing endothelial RCAN1-4 expression inhibited CXCL8 expression but increased endothelial cell network formation (Fig. 78). Thus it is possible that the initial paracrine signalling of CXCL8 could be needed to stimulate ERK1/2 and increase COX-2 and prostaglandin production, as the CXCR2 inhibitor inhibited PG production, and this may be enough to support network formation even without additional endothelial CXCL8 expression. Alternatively, the prostaglandin production, needed for network formation, requiring CXCR2 activation could be regulated by CXCL1 primarily and CXCL8 secondarily. CXCL1 also signals through the CXCR2 receptor therefore PGs will still be produced and able to promote network formation even with a reduction in endothelial CXCL8 expression. Data herein found that antiangiogenic

endothelial ADAMTS1 expression was increased, along with P CM-induced network formation, when endogenous levels of endothelial RCAN1-4 were decreased. Hence, the upregulation of ADAMTS1 and its negative effect on network formation may be counteracted by growth factor signalling such as FGF2 which enhances COX-2 network formation (see Fig. 78). Furthermore, previous research suggests that network formation can be enhanced despite a decrease in proliferation as apoptosis has been shown to enhance endothelial network formation through the pruning of endothelial cells not essential for the structure of the networks (Segura et al., 2002). Research by Segura et al. demonstrated that HUVEC network formation is inhibited by anti-apoptotic gene expression and inhibition of apoptotic proteins (Segura et al., 2002). As RCAN1-4 deficient cells are undergoing apoptosis, this process of cell network pruning could be enhanced in RCAN1-4 deficient cells and facilitate network formation. Whereas on the other hand, the decrease in CXCL8 and increase in ADAMTS1 expression along with activation of apoptosis by hyperactivated calcineurin signalling pathways could overthrow any positive effects that growth factor signalling has on proliferation, leading to cell death.

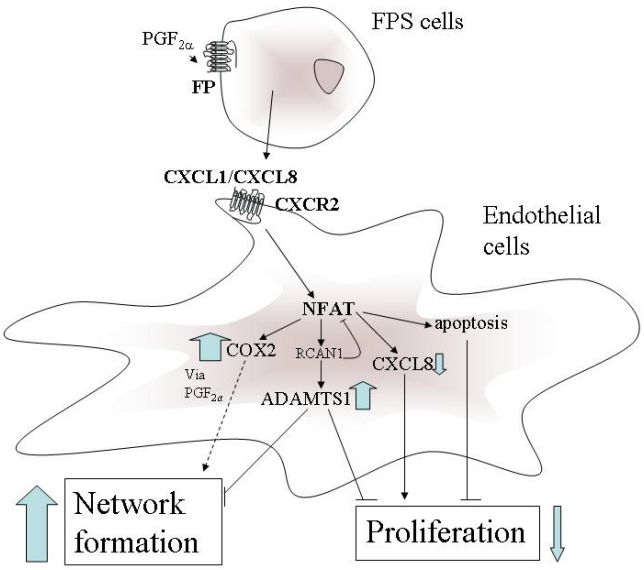


Fig. 78. Possible mechanism for the disregulation of network formation and proliferation in RCAN1-4 deficient cells. In RCAN1-4 deficient cells an increase in NFAT results in increased COX-2 and ADAMTS1 but decrease in CXCL8. The inhibition of proliferation is possibly due to calcineurin hyperactivation and induction of apoptosis. An increase in COX-2 would lead to increased prostaglandin-stimulated network formation.

The data in this chapter discussed so far have revealed evidence to indicate that the expression of RCAN1-4 is crucial for the outcome of growth factor stimulated endothelial cell function. Furthermore, it could be hypothesised that a certain level of RCAN1 is needed to facilitate calcineurin-NFAT induction of CXCL8 therefore, rather than being known as an antiangiogenic protein, RCAN1-4 may more accurately be called a regulator of angiogenesis. Some of the most compelling research for this idea is that, in vivo, RCAN1 knockout mice exhibit a very similar phenotype to the calcineurin knockout mice (Sanna et al., 2006). RCAN1 null mice exhibit hyperactivity in the brain similar to those seen in the calcineurin knockout mice suggesting that RCAN1 facilitates calcineurin signalling in vivo (Sanna et al., 2006). In vitro research suggests that calcineurin signalling may be enhanced by RCAN1 via phosphorylation (Hilioti et al., 2004). Hilioti et al. demonstrate in yeast that the phosphorylation of RCAN1 by GSK3 converts it from an inhibitor to an enhancer of calcineurin (Hilioti et al., 2004). In this way, RCAN1 may be regulated by multiple pathways, the outcome of which will decide if RCAN1 is an activator or deactivator of calcineurin dependent gene transcription.

Alternatively, the bimodal role of RCAN1-4 in the regulation of endothelial cell angiogenic and antiangiogenic gene expression could be a result of the fact that calcineurin has a dual role as an activator and deactivator of transcription signals (Im and Rao, 2004). The role of calcineurin in inactivation of gene transcription may explain the increase in ADAMTS1 seen with the increase in RCAN1. For example, previous research has shown that protein phosphatases, such as PP2A and calcineurin, may dephosphorylate gene transcription silencers known as histone deacetylases (HDACs) thereby promoting their translocation to the nucleus (Martin et al., 2008). Once in the nucleus HDAC binds histones in specific regions of the DNA and deacetylates them thus inhibiting gene transcription (Martin et al., 2007). HDAC6 has been found to prevent the expression of ADAMTS1 in human lung carcinoma cells (Chou and Chen, 2008). HDAC inhibition can also increase the expression of RCAN2 in HUVECs (Wang et al., 2008). Additional regulation may

take place involving 14-3-3, a protein binding partner which can bind phosphorylated HDACs and promote their nuclear export or prevent nuclear import, hence promoting gene transcription (Im and Rao, 2004; Martin et al., 2007). Interestingly, RCAN1 can bind 14-3-3, which relieves its inhibitory effect on calcineurin however, the effect this has on HDAC regulation of gene transcription has yet to be explored (Abbasi et al., 2006). This dual regulatory role of calcineurin and RCAN1 on the activation and deactivation of gene transcription is not unique to these proteins. For example, the calcium/calmodulin-dependent protein kinases (CaMK) are responsible for 14-3-3 phosphorylation which promotes gene transcription (Martin et al., 2007) and recent research has shown that CaMKII can inhibit NFAT-induced transcription by inhibiting calcineurin through phosphorylation (Macdonnell et al., 2009). Evidently more research is needed to understand the precise mechanism of RCAN1 gene transcription and the cross talk between RCAN1-4 and calcineurin signalling.

In addition RCAN1 may act independently of calcineurin and NFAT to regulate endothelial cell functions by either transcription independent or dependent mechanisms. For example, RCAN1 was found to interact directly with integrin αvβ3, inhibiting FAK phosphorylation thereby regulating cell spreading and migration (Iizuka et al., 2004). Alternatively, in HUVECs RCAN 1-4 can bind Raf-1, located upstream of ERK1/2 in angiogenic signalling pathways, however the physiological significance of this has yet to be elucidated (Cho et al., 2005).

In summary, this chapter has investigated the role of antiangiogenic factors ADAMTS1 and RCAN1-4 in the regulation of P CM-induced endothelial cell network formation and proliferation. ADAMTS1 overexpression inhibits P CM-induced endothelial cell network formation and proliferation. In addition, RCAN1-4 overexpression inhibits P CM-induced endothelial cell network formation and proliferation, possibly via the inhibition of CXCL8 expression and the upregulation of ADAMTS1 expression. In contrast, RCAN1-4 deficient cells display an increase in endothelial cell network formation in response to P CM treatment possibly due to increased COX-2 expression. However, RCAN1-4 deficient cells exhibit a dramatic

decrease in endothelial cell proliferation possibly due to altered gene expression of CXCL8 and ADAMTS1 as well as calcineurin hyperactivation activating apoptotic pathways. Data herein demonstrate that the level of endothelial antiangiogenic proteins such as ADAMTS1 and regulators such as RCAN1-4 are crucial for the outcome of P CM-induced endothelial cell function.

7 The in vivo analysis of prostaglandin $F_{2\alpha}$ stimulated angiogenesis.

7.1 Abstract

Data in this chapter investigate the direct action of $PGF_{2\alpha}$ on endothelial F-Prostanoid (FP) receptors, and the indirect, paracrine action of angiogenic growth factors produced as a result of $PGF_{2\alpha}$-FP receptor signalling, in angiogenesis, using two in vivo models. Directly, $PGF_{2\alpha}$ did not increase angiogenesis in an in vivo sponge matrigel angiogenesis mouse model. Indirectly, $PGF_{2\alpha}$-FP signalling increased angiogenic factor expression in human epithelial cells and in mouse stroma but this did not enhance microvessel density. These data indicate that $PGF_{2\alpha}$-FP signalling may not directly regulate angiogenesis however, it may influence angiogenic gene expression and regulate alternative vascular functions in vivo.

7.2 Introduction

In vitro data presented in this thesis have demonstrated that prostaglandin $F_{2\alpha}$ can regulate endothelial cell function through two mechanisms, a direct mechanism and an indirect mechanism. Firstly, via a direct mechanism, the prostaglandin $F_{2\alpha}$ acting through the FP receptor on endothelial cells was found to stimulate endothelial cell network formation but not proliferation. Secondly, via an indirect mechanism prostaglandin $F_{2\alpha}$ treatment of endometrial adenocarcinoma epithelial cells induces the secretion of proangiogenic growth factors FGF2, CXCL1 and CXCL8 which can promote endothelial cell network formation and proliferation via a paracrine action. The P CM-induced effects are, in part, regulated by endothelial cell expression of antiangiogenic ADAMTS1 and regulator RCAN1-4. Additionally ADAMTS1 secreted into the FPS conditioned medium via $PGF_{2\alpha}$-FP signalling could regulate cell function in a paracrine mechanism.

Previous in vivo studies suggest that prostaglandin E_2 can promote angiogenesis via direct and indirect mechanisms. For example, using EP3 null mice, Amano et al.

found that stromal cell signalling of prostaglandin E_2 to the EP3 receptor is crucial for tumour angiogenesis (Amano et al., 2003). In addition, these researchers revealed that VEGF-A growth factor secretion stimulated by PGE_2 treatment is crucial for angiogenesis in a rat sponge model (Majima et al., 2000). Similarly, Jain et al. showed using a matrigel plug assay of angiogenesis that PGE_2 treatment causes an increase in number of CD31 stained vessels (Jain et al., 2008). However, the effect of prostaglandin $F_{2\alpha}$ has not yet been thoroughly investigated.

Experiments were performed using two in vivo models, the matrigel sponge mouse model and the tumour xenograft mouse model, to assess the direct and indirect effect respectively, of prostaglandin $F_{2\alpha}$ on angiogenesis in vivo.

7.2.1 The direct effect of $PGF_{2\alpha}$ on angiogenesis in the matrigel sponge mouse model.

The matrigel sponge mouse (or rat) model has been extensively used by researchers to assess the proangiogenic or antiangiogenic effect of soluble compounds or cells of interest in vivo (Akhtar et al., 2002; Auerbach et al., 2003; Kyriakides et al., 2001). Therefore, the matrigel sponge mouse model (sponge model) was used to examine the effect of $PGF_{2\alpha}$ treatment on vascular formation in vivo. The matrigel sponge mouse model involved the injection of $PGF_{2\alpha}$ directly into subcutaneously implanted matrigel sponges. This experiment was carried out in the laboratory of Dr. P Hadoke. After 21 days of implantation and treatment in the mice, the sponges were removed and vascular density was assessed with the Chalkey vessel count on CD31 stained sponge sections (Fig.79). This assessment of angiogenesis has been used since the 1940's as a reproducible method of measuring microvascular density (MVD) (Chalkey, 1943).

Although there was a dramatic difference in redness of the sponges from the $PGF_{2\alpha}$ treatment compared to the vehicle control treatments from the same mice, which was visible by eye on excision but not after paraffin embedding, no significant difference

in MVD was found between any of the treatments after Chalkey counting (Fig.80). Participants from Group 1 untreated sponges and vehicle treated sponges had very similar MVD counts (Fig.80) suggesting that vehicle treatment had no effect on angiogenesis. Two participants out of the five mice in Group 2, 100nM $PGF_{2\alpha}$ and vehicle treated sponges, showed a significant increase in MVD in the $PGF_{2\alpha}$ treated sponges however, as there was no difference in the other three participants there was no significant difference between groups overall (Fig.80). Immunohistochemical analysis of sponge sections for various growth factors including FGF2, CXCL1 and COX2 showed no observable differences in immunoreactivity from sections between the groups and treatments (data not included). Similarly, QPCR analysis of angiogenic mRNA expression with RNA extracted from 20 sponge halves embedded in RNAlater showed no conclusive differences in mRNA expression between treatments (data not included).

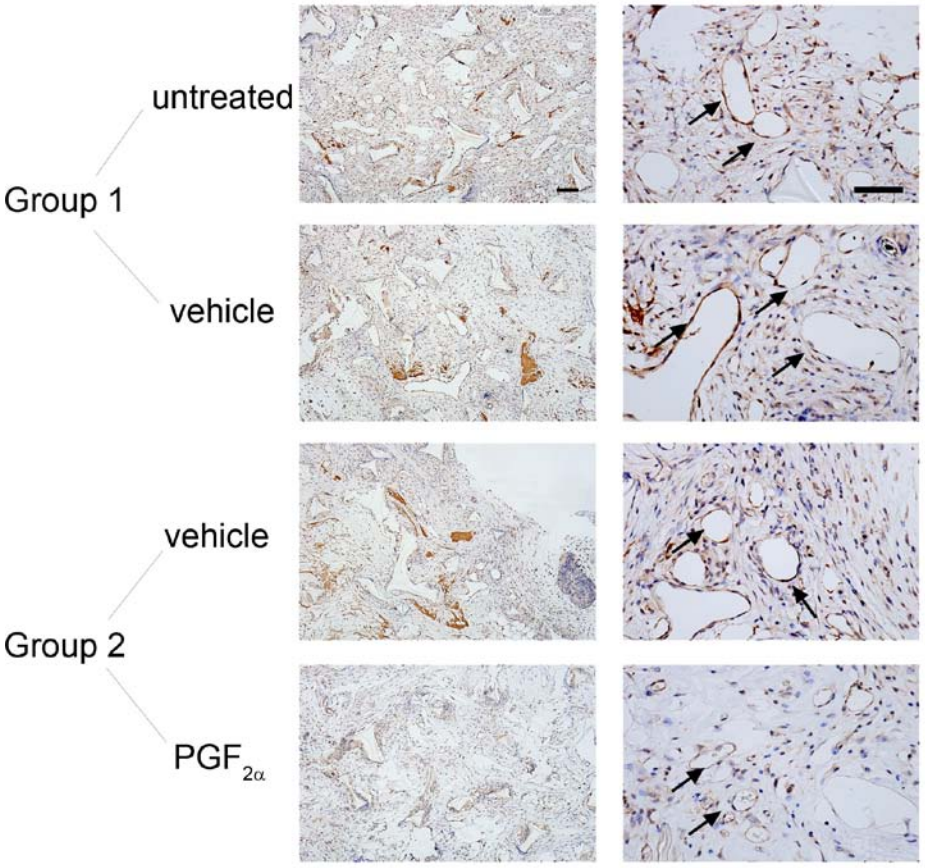


Fig.79.Immunohistochemistry of CD31 in mice matrigel sponge implants. Mice were implanted subcutaneously with matrigel sponges in the left and right flanks and were treated in two groups for 21 days. Group 1 implants were untreated or treated with vehicle. Group 2

implants were treated with vehicle or 100nM $PGF_{2\alpha}$. Immunhistochemical analysis was performed, on sections from five sponges of each treatment, with an antibody for endothelial cell marker CD31. Black bar represents 50µm.

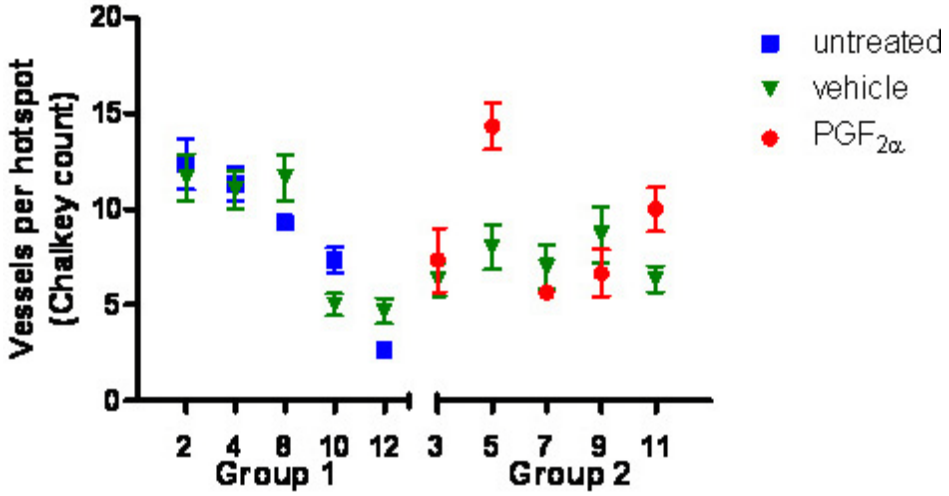


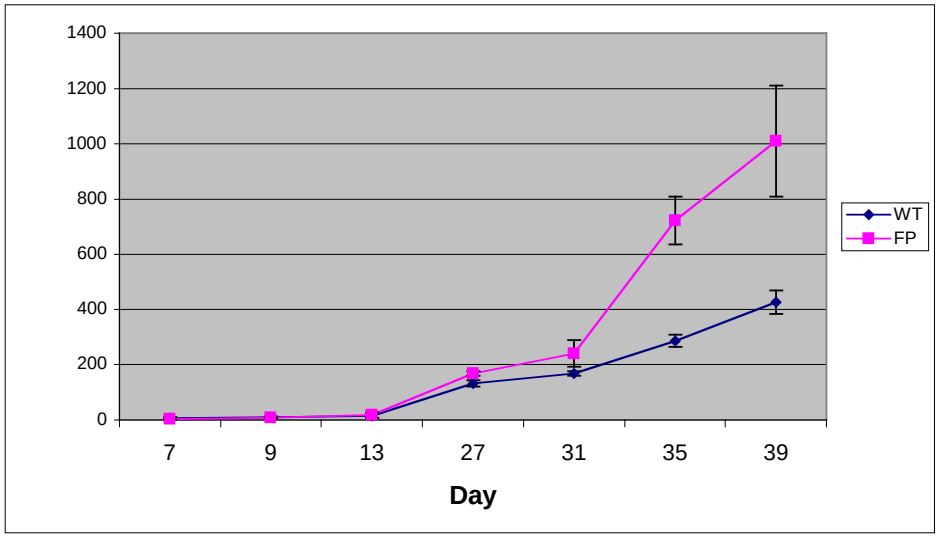
Fig.80. Chalkey vessel counts in mouse matrigel sponge implants. Mice were implanted subcutaneously with matrigel sponges in the left and right flanks and were treated in two groups for 21 days. Chalkey count data Group 1 (even groups) implants were untreated (blue squares) or treated with vehicle (green triangles). Group 2 (odd groups) implants were treated with vehicle (green triangles) or 100nM $PGF_{2\alpha}$ (red circles). A Chalkey graticule was used to count vessels in three hotspots from each sponge implant. Five sponges from each treatment were analysed. Results are displayed as mean ±SEM vessels per hotspot from each sponge.

7.2.2 Mouse tumour xenograft model.

A mouse tumour xenograft model was used to investigate the indirect role of FP signalling on the vasculature. The tumour xenograft model involves injection of cells with tumourigenic potential, such as a cancer cell line, into mice with a compromised immune background to allow the formation of tumours in vivo (Curwen and Wedge, 2009). Commonly, the tumourigenic cells are injected subcutaneously into the flanks of mice because this allows the tumour growth to be non-invasively monitored visually and with bilateral calliper measurement throughout the course of an experiment. Immuno-compromised mice, such as nude mice lacking a thymus, are used so that the hosts do not reject the foreign cells (Rygaard and Povlsen, 1969). The tumours formed can be extracted from the mice and used to examine proliferation and vessel formation. A frequently used marker of

proliferation is Ki-67 protein which is present in cell nuclei during all proliferative stages of the cell cycle but absent in resting cells (Gerdes et al., 1984). In vitro FGF2, CXCL1 and CXCL8 growth factors are secreted by the epithelial $PGF_{2\alpha}$ -FP signalling, as demonstrated in chapter 4, and these growth factors act in a paracrine manner on endothelial cells to promote network formation and proliferation. In this tumour xenograft model WT and FPS Ishikawa cells injected subcutaneously into nude mice formed tumours. The comparison of WT tumour xenografts to FP tumour xenografts allows an examination of the effects of growth factors secreted from the epithelial cells on vascular formation.

The WT and FP tumour xenograft experiment was carried out by researchers in the laboratory of Dr. Daniel Rosenberg (Univ. of Connecticut Medical Centre, USA). Initial analysis from forceps measurements (Fig. 81A) and KI67 staining (Fig. 81B) indicated that the growth rate of the FP tumours was slightly higher than that of the WT tumours (Fig. 81). Ten WT and ten FP tumour xenografts were subsequently analysed by QPCR and immunohistochemistry. As expected the expression of FP mRNA was dramatically elevated in FP tumours compared to WT tumours since the FP tumours originated from FPS cells stably transfected with FP receptor cDNA (Sales et al., 2005) (Fig. 82A, $P<0.05$). There was a significant increase in COX-2 mRNA expression (Fig. 82B, $P<0.05$) but no significant increase in COX-1 expression (Fig. 82C) in FP tumours compared to WT tumours. Analysis of angiogenic mRNA expression with primers and probes that recognised both human and mouse (total) mRNA showed that FGF2 (Fig. 82D), CXCL1 (Fig. 82E), CXCL8 (Fig. 82F) and IL6 (Fig. 83D) mRNA expression was significantly upregulated in FP tumours compared to WT tumours ($P<0.05$).



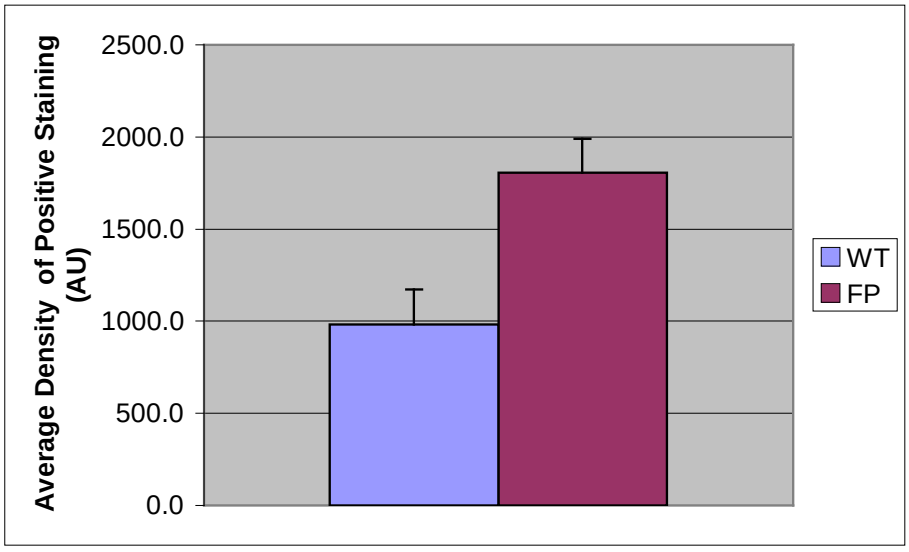


Fig. 81. WT and FP tumour xenografts growth after subcutaneous injection. A, The growth of WT and FP tumours in mice was monitored by measurement with a calibrated forcep throughout their development. B, Ki-67 immunohistochemical staining of WT and FP tumour xenografts. The Ki-67 staining of 3 WT and 4 FPS tumours was quantified using a computer imaging system (Image pro plus).

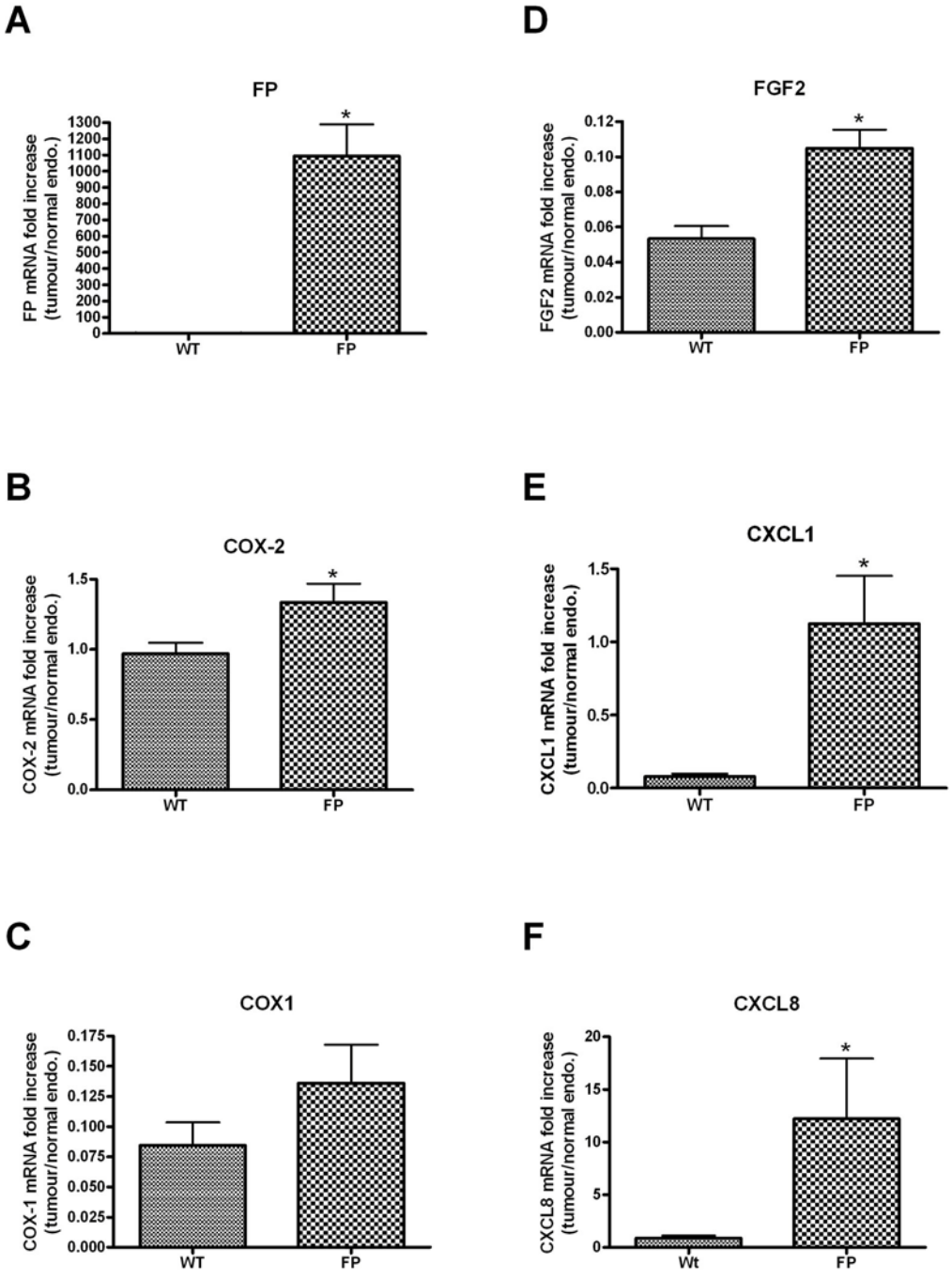


Fig. 82. QPCR of WT and FP mRNA. WT and FP tumour xenograft RNA was extracted and mRNA expression of FP (A), COX-2 (B), COX-1 (C), FGF2 (D), CXCL1 (E) and CXCL8 (F) was analysed using specific primers and probes by RT-PCR (QPCR). Data are expressed as fold increase in tumour xenograft mRNA expression over normal endometrial control tissue mRNA expression. Data are represented as mean ± SEM from ten tumours in each group. (* represents statistical significance from WT tumours; $P<0.05$).

The role of other factors previously found to be involved in angiogenesis was also investigated by QPCR analysis of WT and FP tumours. Surprisingly the potent angiogenic growth factor VEGF-A (Fig. 83A) was not increased in FP tumours compared to WT tumours. Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) have been shown to be involved in angiogenesis in vitro and in vivo (Augustin et al., 2009). Interestingly mRNA expression of Ang1 (Fig. 83B) and Ang2 (Fig. 83E) were both significantly decreased in FP tumours compared to WT tumours ($P<0.05$). Matrix metalloprotease 9 (MMP9) is a protease that is involved in degradation of the extracellular matrix during angiogenesis (Egeblad and Werb, 2002; Roy et al., 2006). MMP9 levels were not significantly different between WT and FP tumours (Fig. 83C). Bcl-2 is a well known antiapoptotic factor involved in tumorigenesis (Cotter, 2009) however, its expression was not found to be increased in FP tumours compared to WT tumours (Fig. 83F).

In chapter 6, data shown indicate that antiangiogenic ADAMTS1 negatively regulates endothelial cell network formation and proliferation induced by P CM. Therefore, the expression of antiangiogenic ADAMTS1 was investigated and found to be significantly decreased in FP tumours compared to WT tumours (Fig. 84A, $P<0.05$). RCAN1 was found to be a regulator of P CM-induced endothelial cell network formation and proliferation and so the expression of RCAN1 in WT and FP tumours was investigated (Fig. 84B). RCAN1 expression was slightly elevated in FP tumours compared to WT tumours but this elevation was not significant (Fig. 84B).

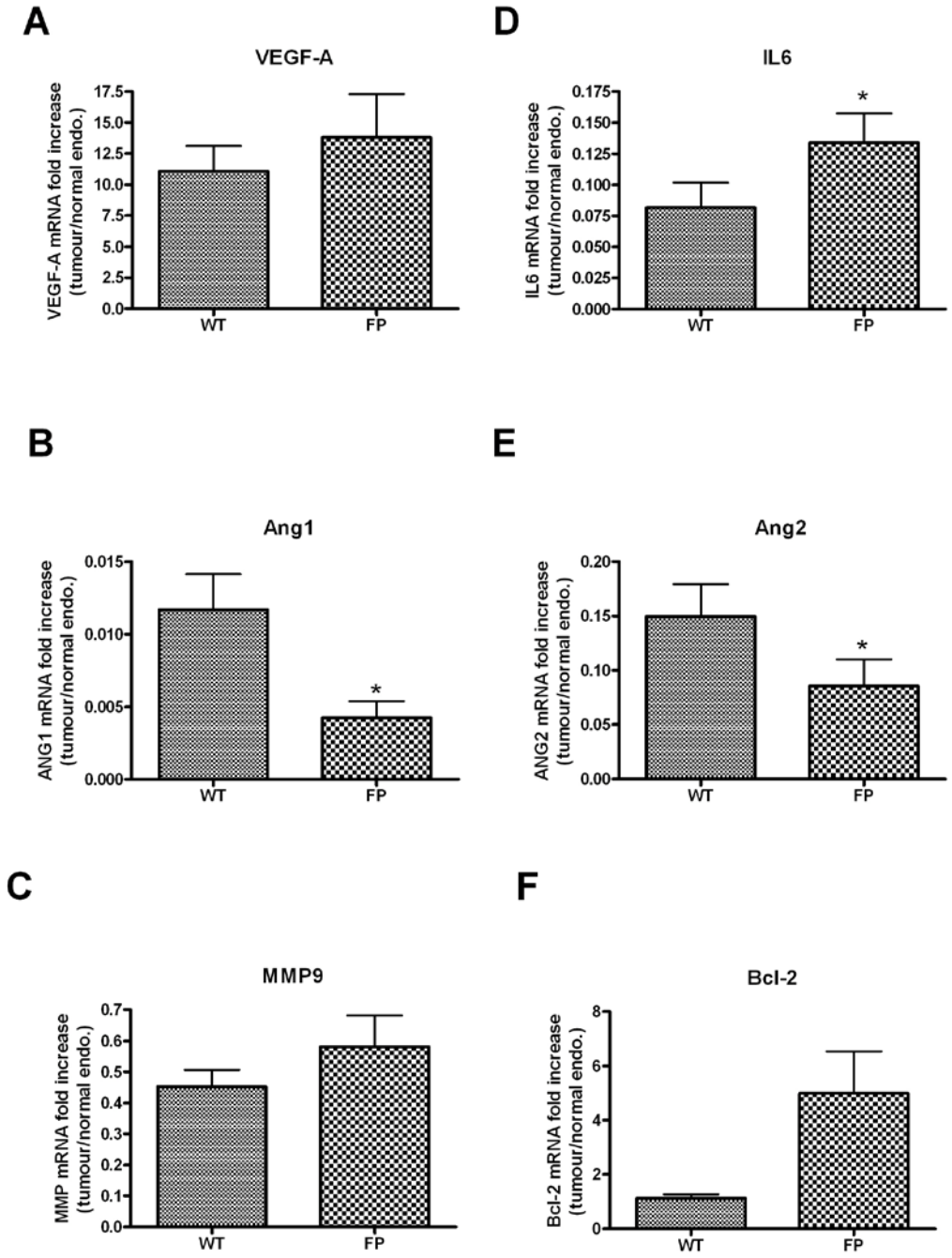


Fig. 83. QPCR of angiogenic factor mRNA expression in WT and FP tumour xenografts. WT and FP tumour xenograft RNA was extracted and mRNA expression of VEGF-A (A), Ang1 (B), MMP9 (C), IL6 (D), Ang2 (E) and Bcl-2 (F) was analysed using specific primers and probes by RT-PCR (QPCR). Data are expressed as fold increase in tumour xenograft mRNA expression over normal endometrial control tissue mRNA expression. Data are represented as mean ± SEM from ten tumours in each group. (* represents statistical significance from WT tumours; $P<0.05$).

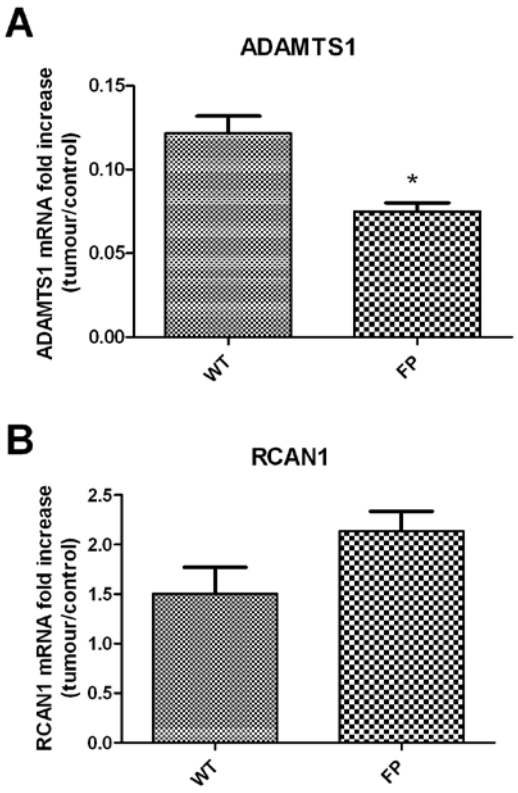


Fig. 84. ADAMTS1 and RCAN1 mRNA expression in WT and FP tumour xenografts. WT and FP tumour xenograft RNA was extracted and mRNA expression of ADAMTS1 (A) and RCAN1 (B) was analysed using specific primers and probes by RT-PCR (QPCR). Data are expressed as fold increase in tumour xenograft mRNA expression over normal endometrial control tissue mRNA expression. Data are represented as mean ± SEM from ten tumours in each group. (* represents statistical significance from WT tumours; $P<0.05$).

Since the in vitro data in chapter 4 showed that CM from FPS cells treated with $PGF_{2\alpha}$ enhanced endothelial COX-2, FGF2, CXCL1 and CXCL8 expression in vitro, primers and probes specific to mouse genes were designed for COX-2, COX-1, FGF2, CXCL1, IL6 and VEGF-A. This allowed the expression of these factors in mouse stromal cells and vascular cells to be investigated in vivo. Human CXCL8 can bind to mouse CXCR2 (IL8RA) and mouse CXCR1 however, the mouse homologue of CXCL8 is uncertain for example, one known ligand of mouse CXCR2 is MIP2 which is more closely related to human CXCL2 (Fan et al., 2007). Due to the uncertainty of mouse CXCL8 homologue, the stromal CXCL8 production was not investigated in this model. A significant increase in mouse COX-2 (Fig. 85A), COX-1 (Fig. 85D), IL6 (Fig. 85C), CXCL1 (Fig. 85E) and VEGF (Fig. 85F) was

found in FP tumours compared to WT tumours. The difference between FP and WT tumour expression of mouse FGF2 was not significant (Fig. 85B).

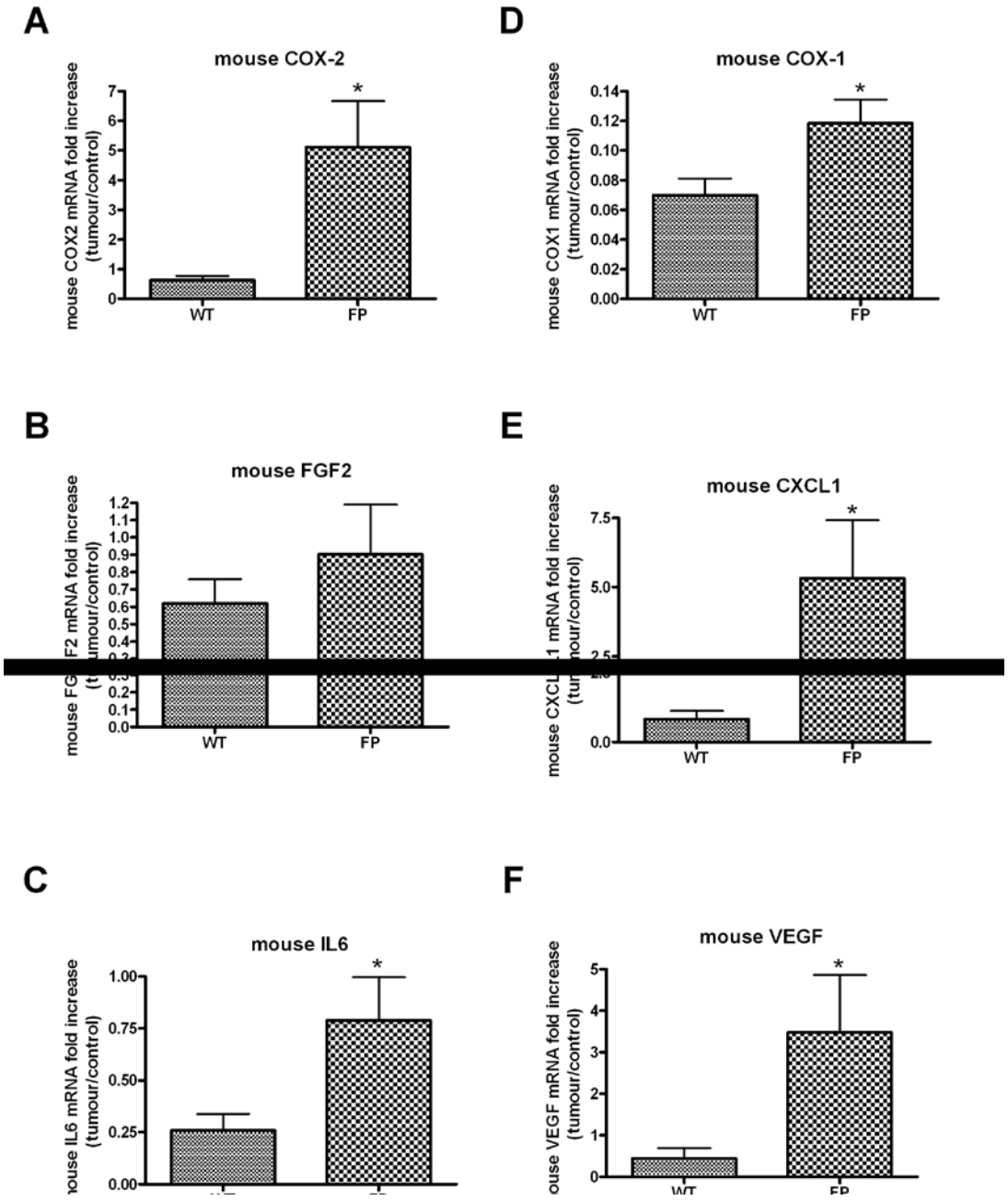


Fig. 85. QPCR of mouse angiogenic mRNA expression in WT and FP xenografts. WT and FP tumour xenograft RNA was extracted and mRNA expression of mouse COX-2 (A), FGF2 (B), IL6 (C), COX-1 (D), CXCL1(E) and VEGF (F) was analysed, using specific primers and probes, by RT-PCR (QPCR). Control was RNA from normal mouse kidney. Data are represented as mean ± SEM from ten tumours in each group. (* represents statistical significance from WT tumours; $P<0.05$).

Sections from WT and FP tumours were stained with an antibody recognising the N-terminus of the FP receptor. The large differences in FP mRNA between the WT and FP tumours indicate that there should be more FP protein in the FP tumours compared to WT tumours. However, immunohistochemical analysis of FP staining did not reveal a clear difference in staining between WT and FP tumours (Fig. 86).

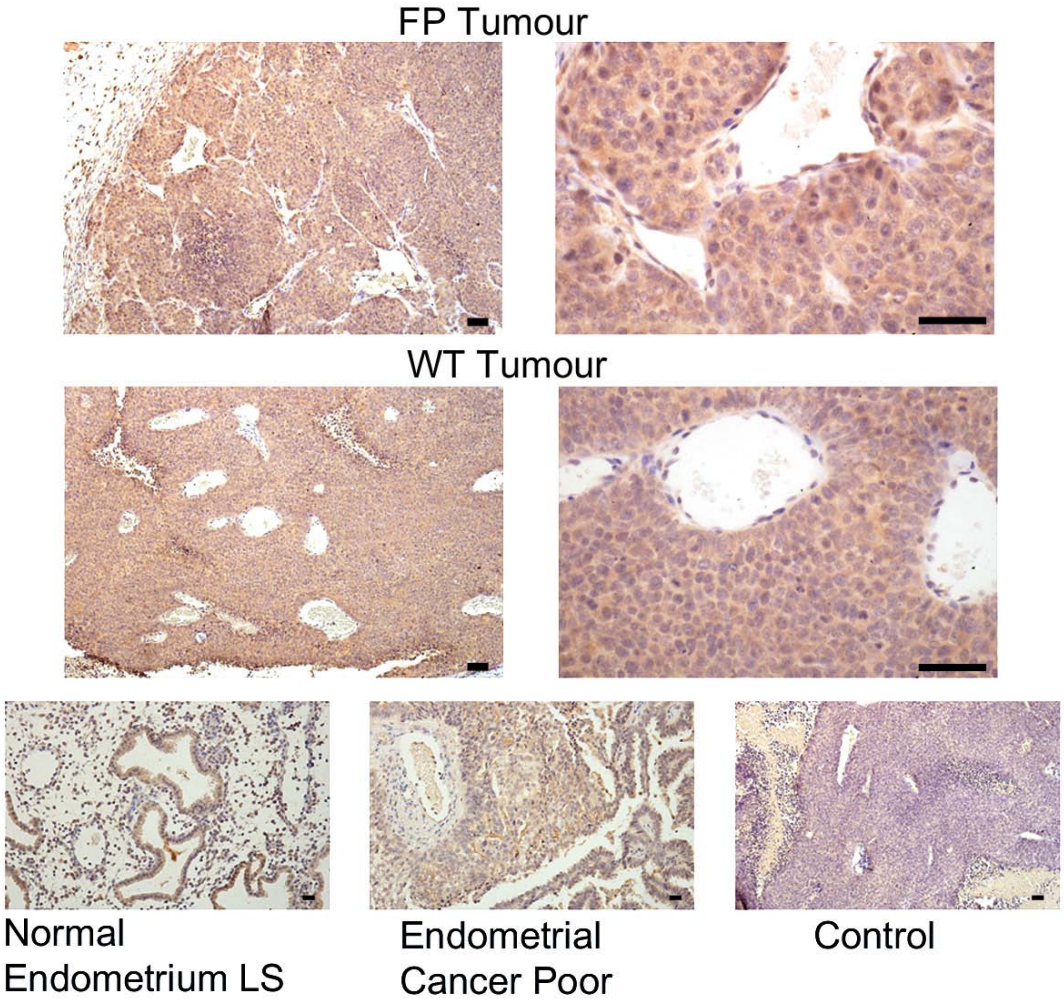


Fig. 86. Immunohistochemical staining of FP and WT tumour xenografts for FP receptor protein. Sections from FP and WT tumour xenografts embedded in paraffin wax were stained for FP receptor protein with a specific FP antibody. Representative photographs at x10 and x40 magnification from FP tumours and WT tumours are displayed above. Sections of normal endometrium late secretory (LS) and endometrial cancer-poorly differentiated (poor) were used as positive controls. Goat IgG was used as a negative control. Black bar represents 50µm.

Both total and mouse COX-2 mRNA expression was increased in FP tumours compared to WT tumours and there was an observable difference in COX-2 staining in FP tumours compared to the WT tumours (Fig. 87).

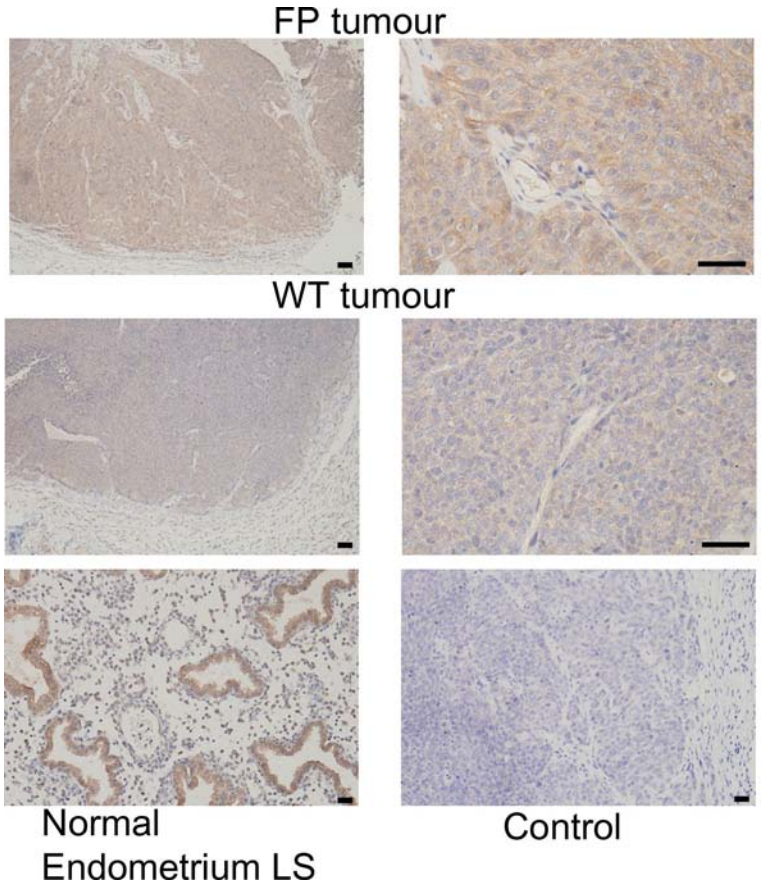


Fig. 87. COX-2 immunohistochemistry of WT and FP tumour xenografts. Sections from FP and WT tumour xenografts embedded in paraffin wax were stained for COX-2 protein with a specific COX-2 antibody. Representative photographs at x10 and x40 magnification from FP tumours and WT tumours are displayed above, taken with Provis, Canon 10D. Sections of normal endometrium were used as a positive control. Goat IgG on a endometrial cancer section was used as a negative control. Black bar represents 50µm.

No observable difference in VEGF-A immunoreactivity was seen between the WT and FP tumours (Fig. 88) similar to the VEGF mRNA expression pattern seen in Fig. 83 in the WT and FP tumours.

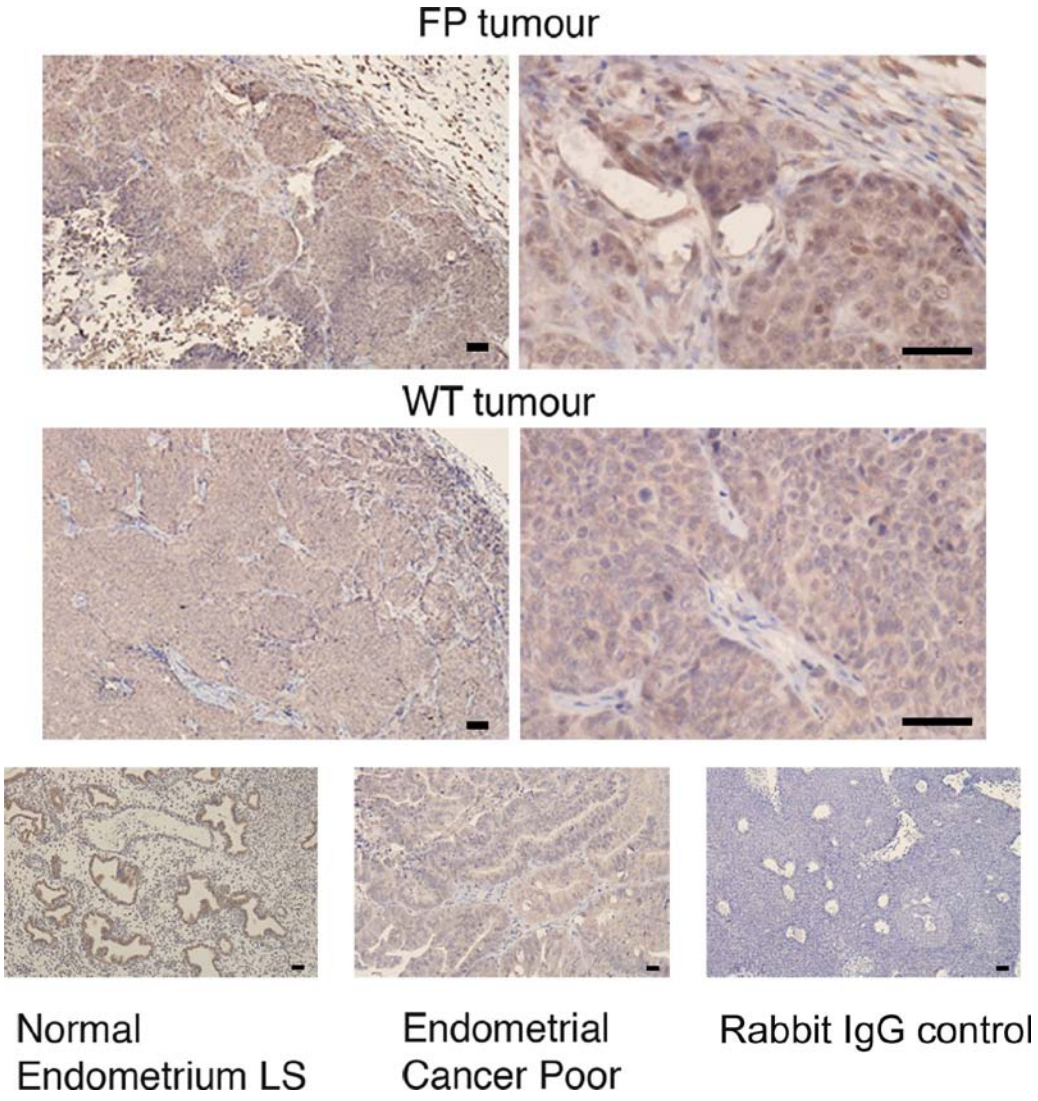


Fig. 88. Immunohistochemistry of VEGF-A staining in FP and WT xenografts. Sections from FP and WT tumour xenografts embedded in paraffin wax were stained for VEGF-A protein with a specific VEGF-A antibody. Representative photographs at x10 and x40 magnification from FP tumours and WT tumours are displayed above, taken with Provis, Canon 10D. Sections of normal endometrium and endometrial cancer were used as positive controls. Rabbit IgG was used as a negative control. Black bar represents 50µm**.**

In accordance with mRNA expression data in Fig. 83, which showed that total CXCL1 mRNA was increased in FP tumours compared to WT tumours, there appeared to be a difference between WT and FP tumour immunoreactivity for CXCL1 (Fig. 89).

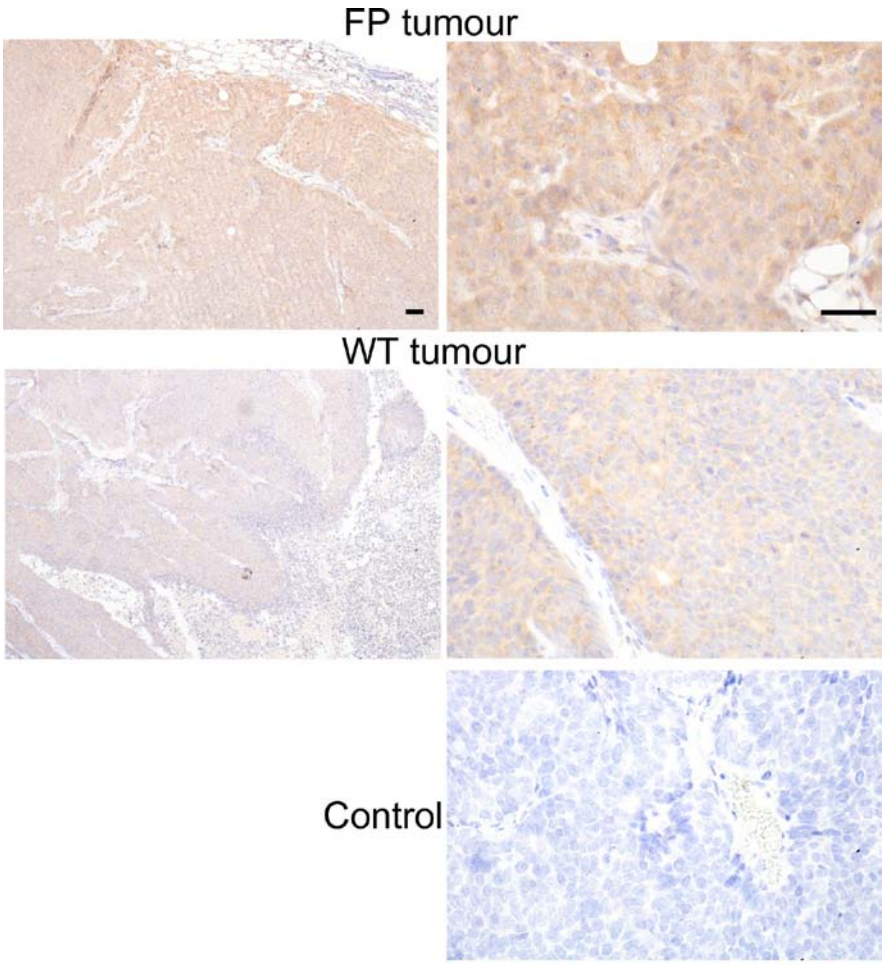


Fig. 89. Immunohistochemistry of CXCL1 in WT and FP tumour xenografts. Sections from FP and WT tumour xenografts embedded in paraffin wax were stained for CXCL1 protein with a specific CXCL1 antibody. Representative photographs at x10 and x40 magnification from one FP tumour and one WT tumour are displayed above. Goat IgG was used as a negative control. Black bar represents 50µm.

7.2.3 The role of CXCL8 in tumour xenograft vascular formation.

In vitro data in chapter 4 suggest that CXCL8 is involved in regulating P CM-induced endothelial cell function. In accordance, CXCL8 has been shown to be involved in vascular formation in vivo (Caunt et al., 2006; Li et al., 2003). Previous data in this chapter shows that human CXCL8 was upregulated in vivo in FP tumour xenografts compared to WT tumour xenografts (Fig. 82) confirming in vitro data. Therefore, to investigate the role of CXCL8 in blood vessel formation in FP tumours compared to WT tumours, CXCL8 was immunoneutralised from the FP tumour xenografts using a specific CXCL8 antibody.

These tumour xenograft experiments were performed by Dr Catalano and Dr Wilson in our laboratory. As observed previously, there was an increase in CXCL8 expression in FP tumours compared to WT tumours. A stereologer was used to assess CD31 stained sections of the tumours and count number of vessels per mm^2 in the outer region of the tumour including the fibroblast layer. The numbers of small, medium and large vessels were quantified as well as the fibroblast area. No significant difference in the number of small (Fig. 90A), medium (Fig. 90B) or large (Fig. 90C) vessels was found between the WT and FP tumour although there was a trend towards a decrease in large vessel size in FP tumours compared to WT tumours (Fig. 90C). There was no difference in total number of vessels per mm^2 in WT and FP tumours, when results from small, medium and large vessel counts were combined (Fig. 90D). The fibroblast layer around the tumours appeared larger in the FP tumours compared to the WT tumours however; this difference was not significant (Fig. 90E).

Immunoneutralisation of CXCL8 with its antibody from the FP tumours (CXCL8Ab) did not reduce CXCL8 expression compared to FP tumours with IgG control (FP IgG), nor did it affect the expression of any other proteins assessed. An initial count of microvascular density in three tumours from each treatment group indicated that perhaps addition of the CXCL8Ab was affecting MVD. Therefore, MVD was

assessed in all ten tumours from WT IgG, FP IgG and CXCL8Ab treatment groups (Fig. 90). However, no significant difference in small (Fig. 90A), medium (Fig. 90B) or large (Fig. 90C) vessels was found between the CXCL8Ab tumours and FP IgG tumours. There was no difference between CXCL8Ab tumours and FP IgG tumours in total number of vessels per mm^2 (Fig. 90D). The fibroblast layer area was not different in CXCL8Ab tumours compared to FP IgG tumours (Fig. 90E).

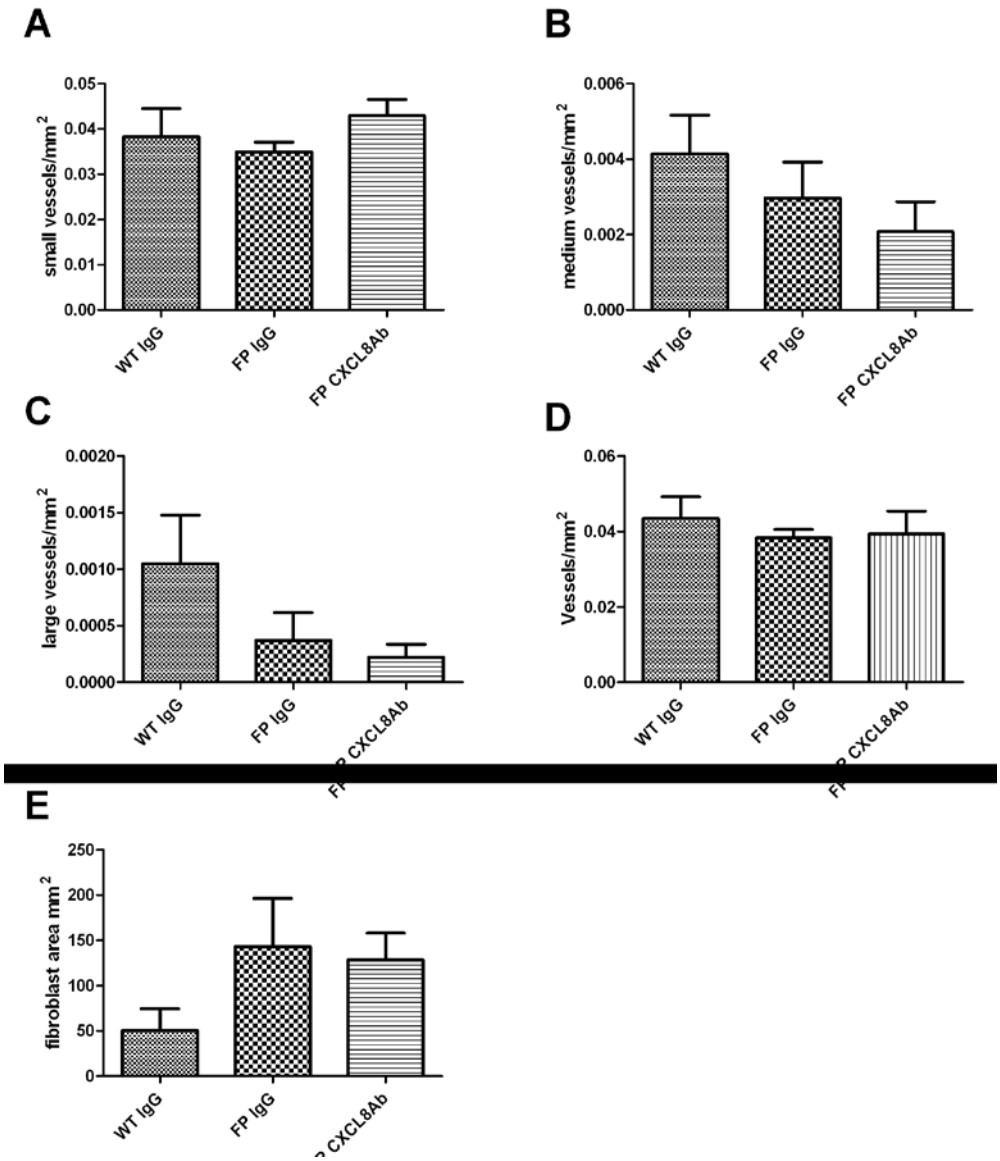


Fig. 90. Microvascular density analysis of WT, FP and CXCL8Ab tumour xenografts. The number of small (A), medium (B) and large (C) vessels was counted from the periphery of CD31 stained sections of WT, FP and CXCL8 Ab tumours using a stereologer microscope and camera (Nikon) at x20 magnification. Total number of vessels (D) was calculated as well as fibroblast area (E). Results are expressed as mean number of vessels per mm^2 ±SEM from ten tumours in each group. (* represents statistical significance)

7.3 Discussion

To investigate the direct and indirect role of prostaglandin $F_{2\alpha}$ on angiogenesis in vivo, two mouse models were used. The matrigel sponge mouse model is frequently used as an in vivo model of angiogenesis (Akhtar et al., 2002). Studies by the laboratory of Majima have shown using the matrigel sponge model in rats, that topical injections of PGE_2 can enhance angiogenesis (Majima et al., 2000). In this chapter, the matrigel sponge mouse model involved the injection of $PGF_{2\alpha}$ directly into subcutaneously implanted matrigel sponges. After 21 days of implantation and treatment in the mice, the sponges were removed and vascular density was assessed with the Chalkey vessel count on CD31 stained sponge sections. The Chalkey count method has been found to be as effective at assessing microvascular density as computational methods (Fox et al., 1995). No significant difference in vessel count was found between treatments however, this method does not take into account any differences in size of the vessels in the sponges. For example, some growth factors such as Angiopoietin 2, affect vessel lumen diameter rather than vessel density (Nasarre et al., 2009). The absence of a difference in vessel number may also indicate that although $PGF_{2\alpha}$ enhances endothelial cell network formation, this is not sufficient for vessel formation in vivo because it does not promote endothelial cell proliferation. A recent study by Herbert et al. demonstrated that inhibition of cytosolic $PLA_2\alpha$, an enzyme involved in arachidonic release, prevented HUVEC proliferation but not network formation (Herbert et al., 2009). Herbert et al. subsequently showed that this $cPLA_2$ inhibition of proliferation was sufficient to block angiogenic tubule formation, and concluded that endothelial proliferation is essential for angiogenesis (Herbert et al., 2009). $PGF_{2\alpha}$ has been shown to have a chemotactic role in enhancing the migration of neutrophils and epithelial cells but size of the granuloma of the sponges appeared to be similar between treatments (Arnould et al., 2001) (Sales et al., 2008a) signifying that there was no increase in infiltrating cell number.

The data presented in this thesis indicates that it is a combination of growth factors and prostaglandins acting in concert that enhance vascular function therefore a

second in vivo model, the mouse tumour xenograft model was investigated. In this model WT and FPS Ishikawa cells were injected subcutaneously into nude mice to allow the formation of tumours. Tumours were excised and analysed by immunohistochemistry and quantitative-RT-PCR. The comparison of WT to FP tumour xenografts allowed the examination of the effects of growth factors secreted from the epithelial cells on vascular formation. This could be likened to comparing the effects of WT V CM and FP V CM on endothelial cell function as seen in chapter 4. Although no significant difference in endothelial cell network formation or proliferation was seen between these treatments in vitro, the in vivo situation is very different in terms of time span and cell-cell interaction. As expected, the levels of FP mRNA expression were significantly upregulated in the FP tumours compared to the WT tumours. The expression of angiogenic growth factors, was also elevated in the FP tumours for example, FGF2, CXCL1, CXCL8 and IL6 were all increased along with COX-2 mRNA. This is in agreement with data from the angiogenic array and ELISA experiments in chapter 4 showing FGF2, CXCL1, CXCL8 and IL6 were secreted in increased quantities into $PGF_{2\alpha}$ treated FPS Ishikawa cell conditioned medium. Therefore, one could infer that the addition of $PGF_{2\alpha}$ to FPS Ishikawa cells in vitro merely enhances the processes that are seen with the autocrine signalling in vivo. A difference in FP receptor protein level was not apparent from the immunohistochemical analysis however; COX-2 and CXCL1 immunoreactive staining appeared to be increased in the FP tumours compared to the WT tumours.

The discrepancy between the in vivo and in vitro data occurred when VEGF-A expression was analysed because it was not upregulated, as would be expected from the in vitro data (Sales et al., 2005), in FP tumours compared to WT tumours. Similarly, there appeared to be no obvious difference between the WT and FP tumours in the immunohistochemical analysis of VEGF-A protein. VEGF-A is a key angiogenic factor whose expression and secretion is upregulated in a variety of cancers and subsequently associated with poorer prognosis of disease through the promotion of tumour progression via angiogenesis and metastasis (Danielsen and Rofstad, 1998; Hainaud et al., 2006; Rofstad and Halsor, 2000; Sales et al., 2005;

Takei et al., 2004; Wei et al., 2003). The in vitro data in this thesis indicated that the balance of growth factors secreted into the P CM was critical as immunoneutralisation of any individual factor from the CM prevented endothelial cell function. In addition, in a previous in vivo tumour xenograft analysis, the immunoneutralisation of VEGF could not be compensated for by the other factors and resulted in a decrease in tumour angiogenesis and metastasis (Rofstad and Halsor, 2000). In endometrial adenocarcinomas, VEGF-A is elevated and has been shown to be regulated by $PGF_{2\alpha}$-FP signalling (Sales et al., 2005). Therefore, the lack of VEGF-A upregulation in the FP tumours could be critical to the use of this experiment as a model for endometrial adenocarcinoma angiogenesis in vivo.

Mouse mRNA expression was examined with specific primers and probes for mouse genes. The mouse mRNA expression was detected in the mouse stroma containing endothelial cells, immune cells and fibroblasts, that have infiltrated the tumour xenografts. Mouse COX-1, COX-2, CXCL1, IL6 and VEGF expression was upregulated in FP tumours compared to WT tumours but there was no change in mouse FGF2 mRNA expression. The upregulation of stromal angiogenic factors in FP tumours supports the in vitro data in chapter 4 showing that paracrine signalling from factors secreted by $PGF_{2\alpha}$-FP signalling in epithelial cells can enhance the expression of growth factor in endothelial cells. Further more, the upregulation of mouse COX-2 indicates that in vivo as seen in vitro, the production of stromal angiogenic factors could involve stromal COX-2 and prostaglandin autocrine signalling. These stromal and epithelial angiogenic factors upregulated in the FP tumours could be enhancing tumour angiogenesis however, the absence of mouse FGF2 upregulation indicates that there is a discrepancy between in vivo and in vitro signalling. This could be explained by the fact that mRNA expression was examined after six weeks of tumour growth in vivo therefore, the time frame of analysis differs greatly from in vitro experiments.

In light of these in vivo data, the specific role of CXCL8 was chosen to be examined in the tumour xenograft model in order to observe the effects on tumour

angiogenesis. CXCL8 and the receptor CXCR2 have all been implicated in tumour angiogenesis using in vivo models (Payne and Cornelius, 2002; Pold et al., 2004; Strieter et al., 2006; Zhou et al., 2005). For example, tumour xenografts from two different prostate cell lines were found to increase angiogenesis in a CXCL8 dependent manner (Moore et al., 1999). Also, a previous report found using a tumour xenograft model of non-small cell lung cancer (NSCLC) that COX-2 overexpressing tumours relied upon the upregulation of CXCL8 to enhance tumour angiogenesis and growth (Pold et al., 2004). In vitro supporting data indicated that this was a PGE_2 and CXCR2 dependent regulation (Pold et al., 2004). In mouse-CXCR2 receptor knockout mice, the growth of human melanoma xenografts is reduced indicating that host CXCR2 and its ligands, including CXCL8, are essential for tumour growth and angiogenesis (Singh et al., 2009).

To examine the role of CXCL8 in the tumour xenograft model, CXCL8 was immunoneutralised from the FP tumours with a specific CXCL8 antibody. Although CXCL8 is not expressed in murine cells, mouse CXCR2 and CXCR1 receptors are expressed, through which human CXCL8 can act (Fan et al., 2007 516). CXCL8 mRNA expression was increased in FP tumours compared to WT tumours. Immunoneutralisation of CXCL8 has been shown in various mouse models of tumourigenesis to decrease vessel density (Inoue et al., 2000; Kim et al., 2001). Examination of mRNA expression as with the previous WT and FP tumours showed that addition of the CXCL8 antibody had no effect on mRNA expression of any of the angiogenic genes of interest (data not shown). In addition, tumour growth rates examined throughout the experiment were not affected by CXCL8 immunoneutralisation.

No difference in microvessel density, assessed from CD31 stained vessels, was found between WT, FP or CXCL8 immunoneutralised tumours suggesting that vascular function is not affected by $PGF_{2\alpha}$-FP signalling to CXCL8 in this model. One theory for this, being explored by Dr. Catalano and Dr.Wilson, is that the WT cells implanted in vivo upregulate their basal FP receptor levels so that the levels of FP

receptor in WT cells are similar to FPS cells prior to xenografting (Catalano et al. unpublished data). This would suggest that sufficient growth factors are being produced to regulate angiogenesis in WT tumours, and elevating the expression further in FP tumours does not benefit tumourigenesis. In support of this theory, microRNA against the FP receptor, added to FP tumours to decrease FP receptor levels results in a decreased number of small vessels (A.Wallace, PhD thesis 2009). This indicates that the FP receptor may play a role in regulating vessel size during angiogenesis in vivo. Two angiogenic factors that have previously been found to play a role regulating tumour vessel size are angiopoietin 1 (Ang 1) and angiopoietin 2 (Ang2) (Augustin et al., 2009). A recent in vivo experiment by Nassare et al. demonstrated that stromal expression of angiopoietin 2 regulates vessel size and tumour growth (Nasarre et al., 2009). In the first WT and FP xenograft in this chapter, the levels of Ang 1 and Ang 2 were found to be downregulated in FP tumours compared to WT tumours however, the levels of stromal angiopoietins were not investigated due to the availability of mouse specific primers and probes. Perhaps investigating the levels of stromal Ang1 and Ang2 could shed some light on vascular morphogenesis within the FP tumours however, angiopoietin expression is inherently linked to time span and examining the expression of angiogeneic proteins at one time point may provide mRNA information which could be open to misinterpretation.

Although CXCL8 immunoneutralisation had no effect on tumour microvascular density, it is possible that the immunoneutralisation of CXCL8 had an effect in the tumour xenografts that affected a different physiological process other than microvascular density investigated in this study. For example, a recent study by Wallace et al., showed that CXCL1 Ab immunoneutralisation from FP endometrial adenocarcinoma tumour xenografts, significantly reduced neutrophil infiltration into the FP CXCL1Ab tumours compared to FP tumours treated with Goat IgG control (Wallace et al., 2009). This indicates that $PGF_{2\alpha}$-FP signalling can stimulate the migration of immune cells towards a tumour. Also, prostaglandin $F_{2\alpha}$ is known to be secreted by HUVECs (Watanabe et al., 1997) and other endothelial cells (Arnould et

al., 2001; Shirasuna et al., 2008) and this endothelial $PGF_{2\alpha}$ has been shown to induce the migration of neutrophils towards endothelial cells (Arnould et al., 2001). Therefore, it is possible that stromal $PGF_{2\alpha}$ stimulated by paracrine $PGF_{2\alpha}$-FP signalling in epithelial cells, may directly enhance stromal cell infiltration. Recently, Sales et al. found that CXCL8 immunoneutralisation from the FP tumours significantly reduced tumour epithelial cell proliferation but increased stromal cell infiltration compared to FP IgG tumours (Sales, 2009).

In addition, there appeared to be a slight difference, although not significant, in fibroblast layer size around the tumours. This could be due to the way in which the tumours were excised or it could be that $PGF_{2\alpha}$-FP signalling is attracting the infiltration of fibroblasts. Another possible chemoattractive factor is ADAMTS1 which was recently shown to stimulate the influx of fibroblasts into xenograft tumours (Rocks et al., 2008). Although ADAMTS1 is not upregulated in FP tumours compared to WT tumours, in vitro studies show it is upregulated in endothelial cells by $PGF_{2\alpha}$-FP signalling (chapter 6) suggesting that as seen by Rocks et al., stromal ADAMTS1 could promote the influx of fibroblasts (Rocks et al., 2008).

In vitro and in vivo evidence suggests that CXCL1 and CXCL8 influence the invasiveness of certain tumours. For example, increased CXCL1 expression was associated with increased invasiveness of glioma tumours (Zhou et al., 2005). Bcl-2 was elevated although not significantly in FP tumours compared to WT tumours and research by Warner et al. demonstrated that endothelial CXCL1 and CXCL8 expression induced by Bcl-2 upregulation, enhanced head and neck tumour cell invasion through a matrigel layer in vitro, which corresponded to a increase in Bcl-2 expression and correlated with head and neck tumour invasion in vivo (Warner et al., 2008). This suggests that the invasiveness of FP tumour xenografts is a further avenue for research.

In summary, these in vivo tumour xenograft data support the role of $PGF_{2\alpha}$-FP paracrine upregulation of angiogenic factors in endothelial cells. However, in vivo

matrigel sponge mouse data together with the previous in vitro data would suggest that the role of $PGF_{2\alpha}$-FP signalling in inducing angiogenesis may be limited by its inability to induce endothelial cell proliferation. Therefore, it could be the combination of epithelial growth factors signalling together with $PGF_{2\alpha}$-FP receptor signalling that is needed to an elicit angiogenic response. Hence prostaglandin $F_{2\alpha}$ may not initiate but may potentiate tumour angiogenesis. This research provides evidence to suggest that targeting of the FP receptor in addition to the targeting of growth factor receptors, such as FGFR1, could provide a possible therapeutic avenue in diseases, such as endometrial cancer, where the FP receptor is aberrantly expressed. In accordance with this, recent in vitro and in vivo research suggest that endothelial FP receptor may be involved in vascular function through the regulation of vasoconstriction and vascular hypertension (Errasti et al., 2009; Yu et al., 2009). However, further research is required to optimise the xenograft mouse model of endometrial adenocarcinoma angiogenesis.

8 Summary, conclusions and future perspectives

8.1 The regulation of angiogenic and antiangiogenic proteins by $PGF_{2\alpha}$-FP signalling in epithelial cells.

In endometrial cancer the FP receptor along with angiogenic factors VEGF-A, FGF2, CXCL1 and CXCL8, have been found to be upregulated compared to normal endometrium. A previous microarray analysis of endometrial adenocarcinoma cells, stably expressing the FP receptor to the levels observed in cancer (Ishikawa FPS cells), treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) identified these proteins VEGF-A, FGF-2, CXCL1 and CXCL8 as targets of $PGF_{2\alpha}$-FP signalling. Further in vitro research, using Ishikawa FPS cells, has found that $PGF_{2\alpha}$ signalling via the FP receptor upregulates the secretion of VEGF-A, FGF2, CXCL1 and CXCL8, in an ERK1/2 dependent manner. Therefore, these angiogenic proteins potentially act on endothelial cells in a paracrine manner to regulate endothelial cell function.

In addition to the regulation of proangiogenic proteins, the antiangiogenic protein a disintegrin and metalloproteinase with thrombospondin repeat 1 (ADAMTS1) was also identified as a target for $PGF_{2\alpha}$-FP signalling in the microarray analysis. In this thesis ADAMTS1 expression was found to be upregulated in endometrial adenocarcinoma samples compared to normal endometrium and localised to glandular epithelial cells and endothelial cells. Using Ishikawa FPS cells, as a model system, the upregulation of ADAMTS1 by $PGF_{2\alpha}$-FP signalling was found to be regulated in an ERK1/2 independent manner involving activation of calmodulin and nuclear factor of activated T cells (NFAT). Therefore, the expression and secretion of antiangiogenic ADAMTS1 into $PGF_{2\alpha}$-treated Ishikawa cell conditioned medium (P CM) could potentially regulate endothelial cell function in a paracrine manner. In the context of endometrial adenocarcinoma, ADAMTS1 could regulate vascular remodelling and angiogenesis. This is because ADAMTS1 can inhibit proangiogenic endothelial cell proliferation and network formation as well as promote ECM degradation and endothelial cell sprouting. Additionally, ADAMTS1 could act in a paracrine manner on epithelial cells and fibroblasts to regulate cell migration and

invasion via mechanisms mediating the degradation of extracellular matrix (see section 8.7.5).

These findings, as summarised in Fig.91, indicate that $PGF_{2\alpha}$ regulates the expression of both pro- and antiangiogenic factors via the FP receptor in endometrial adenocarcinoma. In turn, these factors can act in a paracrine manner on endothelial cells to regulate vascular function. An imbalance between pro-and antiangiogenic factors could enhance angiogenesis in endometrial cancers.

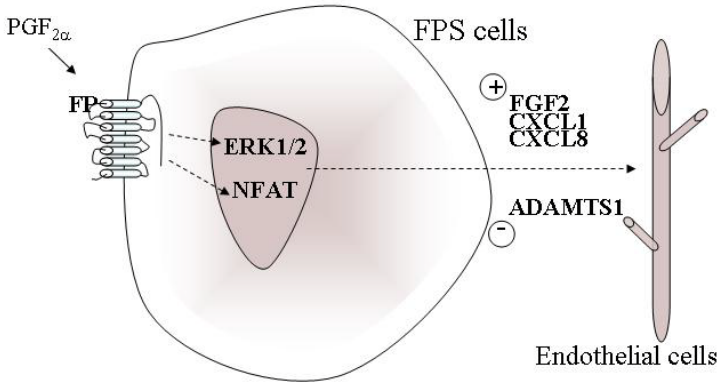


Fig.91. A schematic diagram summarising angiogenic (+) and antiangiogenic (-) factor secretion induced by F-Prostanoid signalling in epithelial cells.

8.2 The paracrine action of FGF2, CXCL1 and CXCL8 on endothelial cell function.

Using an endothelial cell model system of HUVECs incubated with conditioned medium from $PGF_{2\alpha}$-treated Ishikawa FPS cells (P CM), the proangiogenic proteins FGF2, CXCL1 and CXCL8 in the P CM were found to act in a paracrine manner via their respective endothelial receptors, FGFR1 and CXCR2, to promote endothelial cell network formation and proliferation. Activation of FGFR1, via FGF2, and CXCR2, via CXCL1/8, resulted in the upregulation of endothelial FGF2, CXCL1 and CXCL8 creating an autocrine positive-feedback loop. In addition, activation of FGFR1 and CXCR2 regulated COX-2 and FP receptor expression in endothelial cells and promoted the release the $PGF_{2\alpha}$, as shown schematically in Fig. 92.

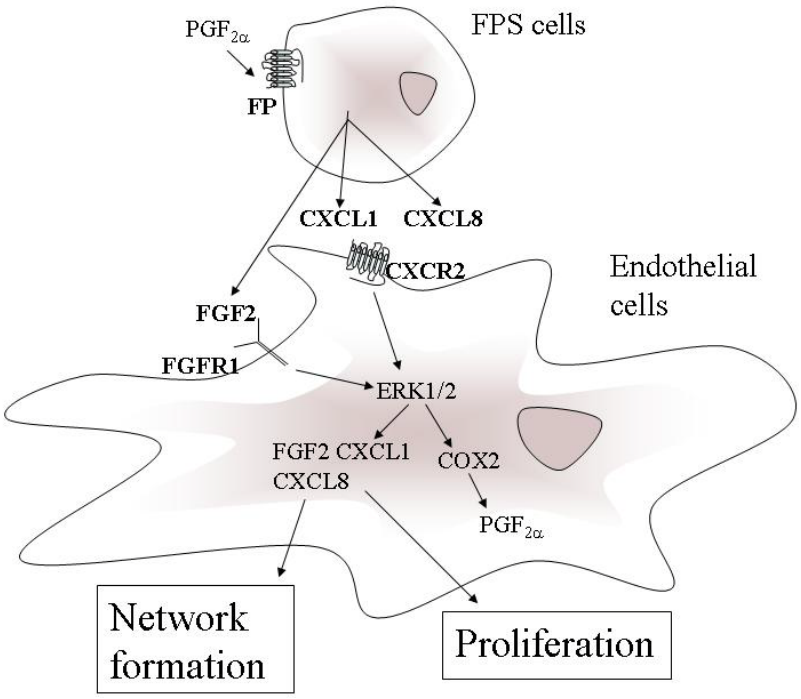


Fig. 92. A schematic representation of mechanism by which P CM regulates endothelial cell network formation, proliferation and $PGF_{2\alpha}$ secretion.

8.3 The role of the FP receptor in P CM-induced endothelial cell network formation and proliferation.

The effects of prostaglandin $F_{2\alpha}$ secretion by endothelial cells , induced by FGF-2 in the P CM, on endothelial cell network formation and proliferation were investigated. $PGF_{2\alpha}$, secreted from endothelial cells after P CM treatment, increased endothelial cell network formation via the endothelial FP receptor but did not regulate endothelial cell proliferation. Further investigations into the intracellular signal transduction pathway mediated by FGF2, revealed that P CM-induced endothelial cell proliferation was regulated in an FGFR1, c-Src, ERK1/2 and mTOR dependent manner. A schematic diagram displaying the divergent signalling pathways controlling P CM-induced endothelial cell network formation and proliferation is shown in Fig. 93.

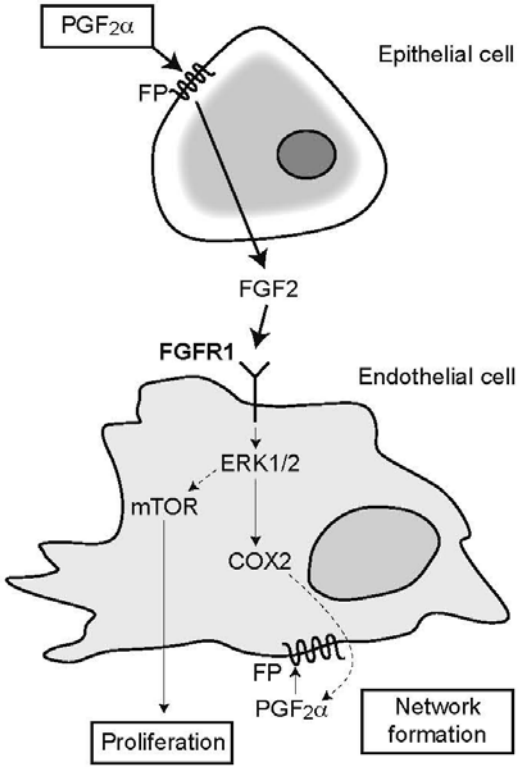


Fig. 93. A schematic representation of P CM-induced endothelial cell network formation and proliferation stimulate via FGF2-FGFR1.

8.4 The role of ADAMTS1 and RCAN1-4 in the regulation of P CM-induced endothelial cell network formation and proliferation.

Antiangiogenic proteins, a disintegrin and metalloproteinase with a thrombospondin repeat 1 (ADAMTS1) and regulator of calcineurin 1-4 (RCAN1-4) were found to be upregulated in endothelial cells after P CM treatment. ADAMTS1 silencing enhanced P CM-induced endothelial cell network formation and proliferation, whereas the overexpression of RCAN1-4 inhibited P CM-induced endothelial cell network formation and proliferation. Overexpression of RCAN1-4 decreased CXCL8 expression and increased ADAMTS1 expression. As summarised in Fig. 94, this suggests that RCAN1-4 may mediate P CM-induced endothelial cell network formation and proliferation by regulating endogenous expression of proangiogenic CXCL8 and antiangiogenic ADAMTS1 in endothelial cells. In contrast decreasing RCAN1-4 expression enhanced endothelial cell network formation but inhibited

endothelial cell proliferation, indicating that a certain level of RCAN1-4 expression is needed for endothelial cell proliferation. These data suggest ADAMTS1 and RCAN1-4 could function as regulators of vascular function by preventing excessive endothelial cell differentiation (network formation) and proliferation in conditions where growth factors are abundant.

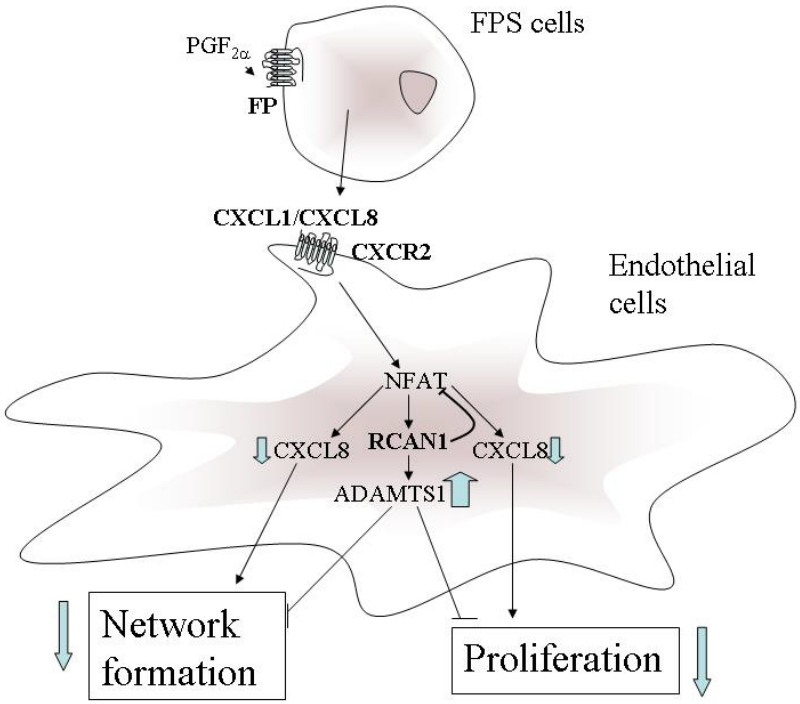


Fig. 94. A schematic represenatation of the regulation of P-CM induced endothelial cell network formation and proliferation by CXCL8, RCAN1 and ADAMTS1.

8.5 In vivo investigations of $PGF_{2\alpha}$-FP signalling in angiogenesis.

The role of prostaglandin $F_{2\alpha}$ in angiogenesis was investigated using two in vivo models. $PGF_{2\alpha}$ did not increase angiogenesis directly in an in vivo sponge matrigel mouse model. In a xenograft mouse model the indirect, paracrine signalling of $PGF_{2\alpha}$-FP increased angiogenic factor expression in human epithelial cells and in mouse stroma but this did not enhance microvessel density. These data highlight the possibility that $PGF_{2\alpha}$ may not regulate angiogenesis directly in vivo; however, it is possible the $PGF_{2\alpha}$ -FP signalling regulates alternative vascular functions such as vasoconstriction which could determine vessel diameter.

8.6 The relevance of $PGF_{2\alpha}$-FP signalling in angiogenesis and vascular function of endometrial adenocarcinoma.

In vitro studies from this thesis indicate that angiogenic factors secreted from endometrial adenocarcinoma cells as a result of $PGF_{2\alpha}$-FP signalling can act in a paracrine manner on endothelial cells to regulate endothelial cell network formation and proliferation, processes involved in angiogenesis. In line with this evidence, endometrial adenocarcinoma endothelial cells have been found to produce more networks in vitro compared to normal endometrial endothelial cells (Du et al., 2008). However, in vivo data in this thesis suggests that angiogenesis assessed by microvessel density (MVD) counts are not affected by $PGF_{2\alpha}$-FP signalling. Early research using the chick chorioallantoic membrane (CAM) assay found that endometrial adenocarcinoma samples grafted to the CAM induced angiogenesis (Palczak and Splawinski, 1989). However, similar CAM and rat corneal angiogenesis assays in which prostaglandins were injected into the CAM or cornea, found that $PGF_{2\alpha}$ could not induce angiogenesis (Barnhill and Ryan, 1983; BenEzra, 1978; Form and Auerbach, 1983; Ziche et al., 1982). In addition, in mice lacking the FP receptor (FP-/- mice), using the retinopathy of prematurity (ROP) model of neovascularisation, no difference was found in levels of vascularisation in FP-/- mice compared to WT mice (personal communication from Dr Ying Yu, 2009, University of Pennsylvania, USA). These data suggest that although $PGF_{2\alpha}$ is secreted by endometrial cancers, it is not the primary factor involved in initiating angiogenesis.

It is therefore possible that $PGF_{2\alpha}$-FP signalling could mediate vascular function by a mechanism independent of angiogenesis that cannot be assessed by MVD counting. Recent in vitro and in vivo data suggests that prostaglandin $F_{2\alpha}$ signalling through the FP receptor may regulate vascular contractility and vascular hypertension (Errasti et al., 2009; Yu et al., 2009). For example, a study by Errasti et al. found that prostaglandin $F_{2\alpha}$ signalling through the FP receptor on endothelial cells, mediates vascular contractility in the human umbilical vein (Errasti et al., 2009). This suggests that the endothelial FP receptor may regulate vessel diameter. In fact, in chapter 7 data showed there was a slight trend towards a decrease in vessel diameter

seen in FP tumour xenografts compared to WT tumour xenografts. Similarly, in vivo studies by Yu et al. showed that FP-/- mice exhibited a decrease in vascular hypertension and blood pressure compared to WT mice (Yu et al., 2009). In addition, studies by Wong et al. on human and hamster renal arteries suggest that an increase in endothelial COX-2, increases endothelial $PGF_{2\alpha}$ secretion, which acts in a paracrine manner on vascular smooth muscle cells (VSMCs), via the thromboxane receptor (TP) to mediate endothelium-dependent contractions (Wong et al., 2009). This paracrine action of endothelial cell $PGF_{2\alpha}$ could also occur in the context of the tumour microenvironment to mediate tumour vessel contractions and regulate intratumoural blood pressure. Intratumoural blood pressure is an important consideration when investigating tumourigenesis as an increase in interstitial pressure, due to rapidly proliferating cells and leaky vessels, can cause collapse of blood vessels leading to hypoxia, if the intratumoural blood pressure is not sufficient. Additionally, intratumoural blood pressure must be considered when investigating blood vessel-mediated delivery of therapeutic drugs to the tumour, as an increase in intratumoural blood pressure may aid therapeutic delivery.

8.7 Future work

Further investigations using more sophisticated technical methods to examine vascular morphology in the WT and FP xenografts could better elucidate the role of the FP receptor in vascular functions of endometrial adenocarcinoma. By employing in vivo bioimaging techniques, vascular perfusion of endometrial adenocarcinoma models could be investigated (McDonald and Choyke, 2003). Vascular permeability is an important consideration when proposing to utilise the blood supply for therapeutic drug delivery into cancers (Fidarova et al., 2008). As prostaglandin $F_{2\alpha}$ secretion from endothelial cells may increase in endometrial adenocarcinoma, the role of endothelial $PGF_{2\alpha}$ in vascular permeability could be investigated in vitro and in vivo. Similarly, as $PGF_{2\alpha}$-FP signalling regulates epithelial cell secretion of vascular endothelial cell growth factor, a vascular permeability factor, and VEGF-A secretion is elevated in endometrial cancer compared to normal endometrium, the paracrine effects of these factors on vascular permeability could be investigated.

In addition, Corrosion casting is one method by which whole tumour xenograft vascular morphology can be examined to assess parameters such as vessel diameter, number of vessel branches and vessel 'dead ends' (Konerding et al., 1999). For example, in an endometrial adenocarcinoma xenograft mouse model, corrosion casting was used to assess the impact of FGF2 on vessel morphology (Konerding et al., 1998). In complement, in vivo bioimaging monitoring the growth of tumours and their blood supply would also provide useful information about the rate and nature of vasculogenesis and vascular morphogenesis (McDonald and Choyke, 2003).

8.7.1 Use of the FP-/- mouse for in vivo corneal angiogenesis assay.

To definitively determine the role of $PGF_{2\alpha}$ and the FP receptor during in vivo angiogenesis, the corneal angiogenesis model could be performed using mice lacking the FP receptor (FP-/- mice). The FP-/- mouse was found to have severe defects in parturition contributing to decreased fertility and recently, the FP-/- mouse was found to have defects in vascular function resulting in vascular hypotension (Yu et al., 2009). Using the FP-/- mice and corneal angiogenesis assay various experimental parameters could be tested including, the effect of $PGF_{2\alpha}$ administered in FP-/- mice compared to WT mice which would investigate the potential of $PGF_{2\alpha}$ to initiate angiogenesis specifically via the FP receptor present on epithelial and endothelial cells. This would identify the specific role of $PGF_{2\alpha}$-FP signalling since $PGF_{2\alpha}$ can also bind to and activate other PG receptors such as the EP receptors as discussed in section 4.4.5 Also by administering $PGF_{2\alpha}$ along with angiogenic proteins such as FGF-2 and VEGF-A, the possibility that $PGF_{2\alpha}$ acting via the endothelial FP receptor could potentiate angiogenesis in combination with other factors could be investigated. Similarly, conditioned medium from WT and FPS Ishikawa cells could be administered onto the mouse cornea so that the corneal angiogenesis experiments could be performed to investigate the role of angiogenic factors, produced by FP signalling in FPS cells, in mediating angiogenesis in vivo.

8.7.1.1 Use of the conditionally deleted PTEN mouse to assess the contribution of the FP receptor in endometrial tumour angiogenesis and vascular function.

Recently, a model involving the conditional deletion of PTEN from the mouse uterus was used to investigate the initiation and progression of endometrial cancer (Daikoku et al., 2008). Endometrial cancer developed in 100% of the conditionally deleted PTEN mice (Daikoku et al., 2008). An increase in COX-2 expression was seen in the early stages of tumour formation suggesting the possible involvement of prostaglandins (Daikoku et al., 2008). A previous study showed that $PGF_{2\alpha}$-FP signalling in Ishikawa FPS cells upregulates COX-2 expression and $PGF_{2\alpha}$ release to promote a positive feedback system (Jabbour et al., 2005). Hence it is plausible that conditional deletion of PTEN and the increase in COX-2 expression could increase the secretion of $PGF_{2\alpha}$ thereby promoting tumourigenesis and mediating vascular function via mechanisms described in this thesis. As the FP receptor is upregulated in endometrial cancer and may be involved in promoting the progression of endometrial cancer (Sales et al., 2005), the contribution of the FP receptor could be investigated in vivo by mating FP-/- mice with conditionally deleted PTEN mice to generate an in vivo model which could provide information as to whether or not FP receptor expression in the uterus affects either the rate of occurrence or growth rate of PTEN-induced endometrial cancer. As demonstrated by in vitro data in this thesis, the endothelial cell FP receptor may mediate endothelial cell function, therefore the physiological role of the FP receptor in the vascular function of endometrial cancer could be assessed. Vascular morphology of tumours could be assessed, using corrosion casting methods, along with the assessment of vascular perfusion. Additionally, microvessel density and angiogenic factor expression could be investigated by immunohistochemical analysis. Investigations into the role of the FP receptor in vascular morphology and perfusion could provide valuable information to facilitate drug delivery to endometrial adenocarcinomas. In addition, data would highlight whether FP receptor expression is essential in promoting tumour progression, in a PTEN background, of endometrial adenocarcinoma. This could perhaps identify the relevance of using the FP receptor antagonists for

endometrial adenocarcinoma therapy in a situation where PTEN is deleted and the FP receptor is overexpressed. As mentioned above, there is a possibility that endothelial $PGF_{2\alpha}$, secreted when endothelial COX-2 is elevated, can act in a paracrine manner on other PG receptors such as the TP receptors of VSMCs to regulate vascular function (Wong et al., 2009). Therefore the extent to which FP expression is needed for tumour growth may not be as high as expected, as there may be a level of receptor redundancy or compensation occurring.

8.7.2 Integrins as mediators of P CM induced endothelial cell function.

Previous research suggests that integrins play a role in angiogenesis and are implicated as key regulators of endothelial cell migration (Avraamides et al., 2008). Integrins are a family of transmembrane glycoprotein heterodimers consisting of non-covalently bound α and β subunits. The function of each integrins is defined by their subunits for example, the β3 subunit of integrin αvβ3 can interact with VEGFR2 receptor in the endothelial extracellular membrane to mediate VEGF-A binding and VEGFR2 activation, an interaction not found with the β1 subunit (Soldi et al., 1999). Integrins interact with the extracellular matrix to transmit 'outside-in' signals that regulate endothelial cell sprouting from capillaries and adhesion. Previous research found that extracellular matrix component alpha-type IV collagen non-collagenous 1 domains (α3(IV)NC1), is an inhibitor of in vitro endothelial proliferation and network formation and in vivo tumour angiogenesis due to its interaction with integrin α3β1 (Boosani et al., 2007). α3(IV)NC1 interacts with α3β1, but not αvβ3, to prevent hypoxia induced-endothelial COX-2 expression which Boosani et al. suggest is the mechanism by which α3(IV)NC1 inhibits angiogenesis (Boosani et al., 2007). Interestingly, the induction of COX-2, and therefore prostaglandin production, by other integrin α3β1-dependent pathways could also be inhibited by α3(IV)NC1 interaction with α3β1. For example, the role of extracellular matrix-integrin interactions in mediating the FGF2-FGFR1 production of prostaglandin $F_{2\alpha}$ and resulting HUVEC differentiation, induced by P CM treatment is a possible further avenue of investigation. Along this line, a recent study

presented findings that in HUVECs, integrin α3β1 binding to fibronectin induces a p38-MAPKinase intracellular signalling pathway that upregulates COX-2 expression along with subsequent secretion of PGE_2 (Viji et al., 2008). Similarly, HUVEC spreading and migration on vitronectin, mediated by αvβ3, was found to be dependent on COX-2 activation and PGE_2 production (Dormond et al., 2001). Also the αvβ3-mediated activation of ERK1/2 is essential for FGF2 mediated angiogenesis in the in vivo CAM model (Eliceiri et al., 1998). These data indicate that integrins may be involved in mediating the ERK1/2, COX-2-$PGF_{2\alpha}$ pathway induced by FGF2-FGFR1 signalling after P CM treatment.

Alternatively, integrin α9β1 was recently found to directly bind VEGF-A and promote angiogenesis. In addition, α9β1 binds to the N terminus of thrombospondin 1 fragments to promote angiogenesis. Inhibition of α9β1 prevents angiogenesis. It would be interesting to investigate if ADAMTS1, which is a negative regulator of VEGF-A and responsible for the release of TSP1 fragments into the cellular matrix, may interact with or mediate integrin-dependent angiogenesis via integrins such as α9β1.

8.7.3 The role of IP receptor and PPAR in P CM-induced endothelial cell proliferation.

The data in chapter 4 showed that the COX inhibitors inhibit endothelial cell proliferation however, involvement of the EP2 or FP receptors in endothelial cell proliferation was not found. Hence, it is possible that the COX-dependent endothelial proliferation is reliant on an alternative COX product. One possibility is that prostacyclin is the COX product involved in endothelial cell proliferation. The IP receptor has been implicated in the regulation of endometrial angiogenesis (Smith et al., 2006). Prostacyclin treatment can induce angiogenesis and vascular permeability (Pola et al., 2004). For example, in guinea pigs, intradermal injection of VEGF-A, but not FGF2, upregulated PGI_2 expression and increased vascular permeability both of which could be inhibited by the addition of the COX inhibitor Indomethacin (Murohara et al., 1998). However, as well as signalling through the IP

receptor, prostacyclin can also signal through PPAR alpha nuclear receptors to upregulate VEGF expression and induce angiogenesis in vivo (Pola et al., 2004). In fact this previous study found using a mouse cornea neovascularisation assay that a prostacyclin analogue which acts only through the IP receptors in the cell can not facilitate angiogenesis in vivo (Pola et al., 2004). This study was recently verified using PPAR-/- mice which exhibit reduced corneal angiogenesis in response to iloprost, a stable prostacyclin analogue (Biscetti et al., 2009). Therefore it is possible that prostacyclin acting through the nuclear PPAR receptor or the IP receptor may be involved in the regulation of P CM-mediated endothelial cell proliferation. Further in vitro investigations to evaluate the role of the IP receptor in endothelial cell proliferation could utilise methods of IP receptor antagonists, gene silencing or IP receptor overexpression in endothelial cells. Additionally, in vivo investigations could involve the use of WT and FP conditioned medium in the corneal angiogenesis assay in PPAR-/- mice to determine whether vascular function, as investigated in this thesis, is mediated via signalling mechanisms that coactivate nuclear receptors such as PPAR.

8.7.4 The role of non-conventional cyclopentanone prostaglandins in COX-regulated endothelial cell proliferation.

An alternative intriguing explanation of the fact that COX inhibitors inhibited endothelial cell proliferation (see chapter 4), although the EP2 or FP receptors were not involved in endothelial cell proliferation (see chapter 5), is that COX-dependent endothelial proliferation could be regulated by non-conventional cyclopentanone prostaglandins (cPGs) such as PGAs and PGJ_2 which may regulate gene transcription without exiting the cell. However, most evidence from macrophages suggests that cPGs are involved in the down-regulation of gene transcription via PPARγ activation (Ricote et al., 1998) and 15d-PGJ_2 has been shown to inhibit rat corneal angiogenesis, HUVEC network formation and proliferation (Xin et al., 1999). On the other hand, HUVEC apoptosis is stimulated by PGJ_2 treatment upregulating p53 in a PPARγ independent mechanism (Ho et al., 2008). Therefore, it is likely that

these cPGs will be antiproliferative rather than proliferative although further studies are needed to confirm their role in P CM-induced endothelial cell proliferation.

8.7.5 The role of ADAMTS1 in endometrial adenocarcinoma.

In this thesis, antiangiogenic ADAMTS1 was found to localise to the glandular epithelial and endothelial cells of endometrial cancer sections. Also, ADAMTS1 was found to inhibit P CM-induced endothelial cell network formation and proliferation. ADAMTS1 is secreted by endothelial cells and controls endothelial cell network formation and proliferation however, ADAMTS1 may mediate other vascular functions such as endothelial cell invasion. For example, Su et al. found that ADAMTS1 was responsible for enhancing endothelial cell sprouting in an in vitro cell model (Su et al., 2008). Additionally, ADAMTS1 can enhance endothelial cell migration (Krampert et al., 2005). A possible further experiment investigating the effect of ADAMTS1 on endothelial cell matrix invasion could be performed by attaching endothelial cells to microcarrier beads, embedding them in a collagen/matrigel matrix and coculturing with WT and FPS cells. In this model, the role of ADAMTS1, both paracrine and autocrine, could be examined with ADAMTS1 siRNA transfection of Ishikawa cells or endothelial cells.

Alternatively, as highlighted in chapter 3, ADAMTS1 is also released from epithelial cells in response to $PGF_{2\alpha}$-FP signalling. Consequently, ADAMTS1 could regulate other aspects of tumourigenesis in addition to vascular function. For example, data by Rocks et al. demonstrated that cancer conditioned medium from ADAMTS1 overexpressing bronchial epithelial tumour cells can regulate the migration of fibroblast cells (Rocks et al., 2008). Similary, Krampert et al. found that ADAMTS1 mediated fibroblast cell migration in an in vivo mouse model of wound healing (Krampert et al., 2005). As ADAMTS1 expression was elevated in endometrial adenocarcinoma and increased by $PGF_{2\alpha}$-FP signalling, the paracrine actions of ADAMTS1 in mediating fibroblast migration of endometrial cancer could be investigated.

8.7.6 Elucidating the mechanism by which RCAN1-4 may regulate ERK1/2 induced gene expression.

It is clear from the data in chapter 6 and previous studies that RCAN1-4 can prevent and enable endothelial cell proliferation perhaps due to the fact that changes in the levels of RCAN1-4 can inhibit and promote gene expression (Hilioti et al., 2004; Sanna et al., 2006). For example, RCAN1-4 overexpression appeared to inhibit calcineurin-NFAT induced gene expression of VEGF-A and CXCL8 but increase ADAMTS1 expression (Hesser et al., 2004). RCAN1-4 may regulate endothelial cell function by transcription dependent mechanism, i.e. through the inhibition of calcineurin-NFAT mediated transcription (Hesser et al., 2004) or transcription independent mechanisms, i.e. through the regulation of second messenger activation by binding Raf (Cho et al., 2005). As Raf is an important molecule involved in the activation of MEK1/2 and therefore ERK1/2, it would be interesting to investigate the role of RCAN1-4 in the regulation of ERK1/2 activation. ERK1/2 is activated by MEK and deactivated by phosphorylation and dephosphorylation events (Raman et al., 2007). ERK1/2 activity can be regulated by the non-specific protein phosphatase 2A (PP2A) (Raman et al., 2007; Van Kanegan et al., 2005). PP2A can enhance ERK1/2 phosphorylation indirectly by dephosphorylating inhibitory sites on Raf (Raman et al., 2007). On the other hand, PP2A can directly dephosphorylate and inactivate ERK1/2 (Van Kanegan et al., 2005). In rat neuronal cultures, the calcineurin inhibitor cyclosporin A (CsA) inhibited ERK2 dephosphorylation after glutamate treatment suggesting that the phosphatase calcineurin (PP2B) may also regulate ERK2 phosphorylation by indirect or direct mechanisms (Paul et al., 2003). Therefore, as hypotheses for further investigations, RCAN-1 may interact with the ERK1/2 pathways in a number of ways by a) interfering with the activation of Raf by PP2A, b) interacting with the PP2A mediated dephosphorylation and inactivation of ERK1/2, or c) inhibiting ERK1/2 dephosphorylation mediated by calcineurin (PP2B).

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